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The Mystery of SPO1 Delayed Early Genes

by

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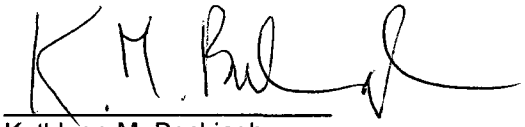

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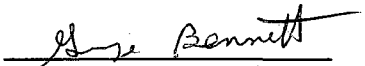

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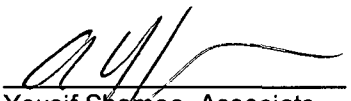

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ABSTRACT

The Mystery of SPO1 Delayed Early Genes

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Genes of the *Bacillus subtilis* infecting bacteriophage SPO1 are classified as early, middle or late genes, based on which sigma factor is used for their transcription. Within the early gene category, there are two subsets of genes, immediate and delayed early (including genes 37-40), which have different temporal expression patterns. Genes 37-40 are shown involved in the shutoff of host macromolecular synthesis. Gene products (gp) 39 and 40 were shown to be lethal when expressed in *E. coli* cells, through an undetermined mechanism. Gp39 was purified under denaturing conditions, but could not be properly refolded.

Genes 2.21, 25.1 and 51 were studied in order to understand how delayed early genes are regulated. Gp51 was partially purified. An additional gene expressed from within gene 51 was discovered. It was determined that SPO1 mutations of genes 2.21 and 25.1 could not be isolated, even when a variety of conditions were tested.

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This dissertation is dedicated to my family, namely my daughter Elizabeth Bin Bin and my husband Peter.

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Ch 1: Background

1.1 SPO1 lifecycle- host takeover

SPO1 is a lytic, virulent bacteriophage that infects *Bacillus subtilis* (*B. subtilis*), a Gram-positive bacterium. When bacteriophages infect their host bacterium, they take over the host's DNA, RNA and protein synthetic abilities, making the host a factory for phage production. The host's synthetic processes are almost entirely shut off, removing competition for phage macromolecule production. The cells then produce the components for phage reproduction, such as phage DNA and phage capsids, which are then assembled into new phage. Lysins are produced to lyse the cells, allowing the phage to burst forth and find new bacteria to infect. The host takeover process is of interest as it requires highly specific mechanisms to allow for high levels of phage biosynthesis while shutting off most host DNA, RNA and protein synthesis. Several mechanisms involved in this process appear to be unique to SPO1.

1.2 Non-SPO1 Phage Host Takeover

When a host cell is infected with a lytic phage, the host's macromolecular synthesis processes are almost entirely shut off, removing competition for phage macromolecule production. Even in a well-studied system like T4, which infects E. coli, the takeover process is not fully understood. As reviewed by Kutter, the shutoff of host translation is known to involve RegB endoribonuclease. However, regb mutants can still efficiently shutoff protein synthesis (Kutter et al., 1994). This indicates that more gene products (gps) must be involved though these are not yet understood. One of the proteins involved in host DNA replication shutoff is the protein Ndd (nuclear disruption), which acts by moving the genome away from the center of the cell and blocking replication (reviewed in Kutter et al., 1994). Meanwhile, the host RNA shutoff mechanism takes advantage of one of the unique qualities of T4. In the T4 DNA, 5-hydroxymethyl cytosine replaces cytosine. The Alc protein prevents transcription from any DNA containing cytosine, shutting off host RNA synthesis specifically. Termination of transcription occurs at specific sites through interaction of Alc with the transcription complex. At the same time as these proteins are acting, T4 directed endonucleases II and IV degrade

cytosine containing DNA. The nucleotides are then used for phage DNA synthesis (reviewed in Kutter et al., 1994).

T7 employs many different mechanisms to shut off host biosynthesis, some similar to T4, some very different. One different mechanism is the production of a T7 specific RNA polymerase (RNAP), allowing T7 to deactivate the host RNA polymerase. It does this though gp0.7 and gp2. Gp0.7 is a kinase which phosphorylates RNAP, while gp2 is an inhibitor that binds to RNAP. Other mechanisms used involve mRNA stability and protein stability. One mechanism similar to a T4 mechanism is the degradation of host DNA. A variety of phage gene products act to prevent restriction enzymes from degrading T7's DNA, while the host's DNA is degraded (Kruger and Schroeder, 1981).

As currently understood, SPO1 does not appear to use the same mechanisms. For example, in SPO1, there is no host DNA degradation (Yehle and Ganesan, 1972). This is surprising as SPO1 uses hydroxymethyl uracil instead of thymidine in its DNA, which would allow for clear distinction between SPO1 and host DNA. As discussed in more detail throughout this work, many of the mutants of shutoff genes have unique phenotypes, not seen in other phage. SPO1 also does not

produce its own RNAP, though it does produce its own sigma factors, discussed in detail below. Information about unique shutoff mechanisms and genes may be of practical use in designing new antibiotics.

1.3 SPO1 Gene Classification

SPO1 gene regulation heavily involves RNAP sigma factors. A sigma factor is a subunit in RNAP which confers specificity to RNA transcription. In SPO1, genes are classified based on which sigma factor is required for their transcription: early genes use σA (a host sigma factor), middle genes use gp28 (an SPO1 early gene), and late genes use gps33/34 (an SPO1 middle gene) (see Table 1.1). SPO1 infection begins with the transcription of early genes by σA, the B. subtilis sigma factor synthesized while the host is growing in exponential phase. These early genes are responsible for most of the host takeover process. One of these early genes specifies the sigma factor gp28, which causes transcription of the middle genes, which are responsible for phage DNA replication. Genes 33 and 34 encode yet another sigma factor, which leads to transcription of late genes. Late genes code for the structural and morphogenic phage proteins (Fujita et al., 1971; Gage and Geiduschek, 1971; Losick and Pero, 1981; Talkington and Pero, 1977).

Table One: Gene Categorization

	Immediate Early	Delayed Early	Middle
Expression Time (30C)	1-10 minutes	10-20 minutes	10-30 minutes
Sigma factor	B. subtilis A	unknown	SPO1 gp28
Representative genes	48-49, 53-55, 56-58, 44, 50-51	28, 37-39, 40	52, 31, TF1

Table 1: Summary of Gene Classifications: Summary of the properties of Immediate Early, Delayed Early, Middle Genes, listing when the bulk of expression occurs, sigma factor used and representative genes.

1.4 Delayed Early Genes

While some genes can easily be classified as early, middle or late, other genes are more difficult to define. Early genes, for example, can be split into two categories: immediate early and delayed early. Genes 27-28 and 37-40 are classified as delayed early genes. These genes are expressed later than immediate early genes, but, like the immediate early genes, do not rely on gp28. The role of genes 37-40 is not well understood, but it is believed that they are involved in the shutoff of host macromolecular synthesis. Understanding the role of the delayed early genes is critical for understanding the takeover process.

The regulation of immediate and delayed early genes is currently not understood. Both immediate and delayed early genes appear to use the same promoter, though there is some evidence, discussed below, that a different promoter may be required for delayed early gene expression. Currently, no transcription factors have been found that fully explain the differences in the timing of delayed early and immediate early expression. Genes 44 and 51 have already been shown to be involved in this process, but they do not play a causative role (Sampath and Stewart, 2004). Genes 25.1 and 2.21, recently discovered with the sequencing of the SPO1 genome (Stewart et al, 2009), are potentially involved in the regulation of immediate and delayed early genes.

1.5 Operon 37-40

Genes 37-40 are delayed early genes, located in the terminal redundancy, in the host-takeover module, where 24 genes believed to be involved in host takeover are located. Host-takeover genes must be expressed early in infection, which fits with either immediate or delayed early genes. The promoter for the operon is very active, and the ribosome binding sites for each of the four genes are strong (Stewart et al., 1998). The 4 genes encode for protein of various sizes, from 31 amino acids for gp37, 190

amino acids for gp38, 255 amino acids for gp39 to 350 amino acids for gp40. Currently, BLAST searches using both amino acid and nucleic acid sequences have shown no significant matches between the four SPO1 gene products and the GenBank database, meaning that in order to make any hypotheses regarding their function, experimental data must be obtained.

1.6 Genes 44, 50, 51

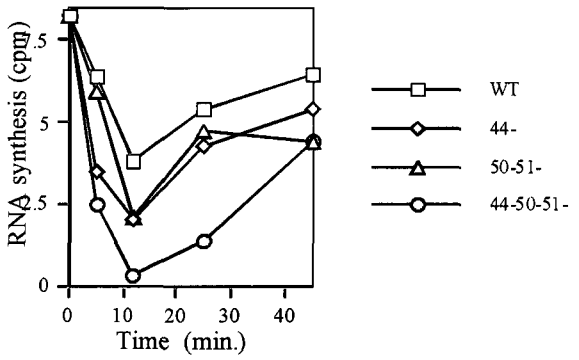

Figure 1.1: Shutoff of host RNA synthesis. Rates of RNA synthesis were measured, by pulse-labeling with [5-^{3}H] uridine, as a function of time after infection by the indicated strains. The graph shows TCA-precipitable kcpm; i.e. cpm X 10^{-3} (Stewart and Sampath, 2004).

Experiments using nonsense mutants of genes 44 and 51 show that these gene products regulate the shutoff of host RNA synthesis. These nonsense mutants were created by mutating a lysine early in the gene to a stop codon. In the 51 nonsense mutant, gene 50 is also mutated. This is due to the 8 base

overlap between genes 50 and 51. Due to gene 50's small size, specifying only 23 amino acids which form a single membrane spanning domain, we are focusing on gene 51. As shown in figure 1.1 (Sampath and Stewart, 2004), nonsense mutants of either gene 44 or 50/51 show decreased levels of total RNA (host and phage synthesis when compared with wild type (wt)). This indicates that SPO1 is more effective at shutting off host RNA synthesis without these genes. Therefore, it is likely that these genes normally act to slow the shutoff process, indicating a regulatory role.

In addition to genes 44 and 51's regulatory role, the mutants show that these genes are not essential for regulation of delayed early genes. The mutants were assayed looking at the RNA levels of individual genes (figure 1.1) (Sampath and Stewart, 2004). Strains of 44^- and $50^-/51^-$ show higher expression compared to wild type for immediate early, delayed early and middle RNA expression. This eliminates genes 44 and 51 as the causative factor for delayed early gene expression, as a nonsense mutant of the causative factor would result in a knockout of delayed genes, not an increase in expression (Sampath and Stewart, 2004). While these gene products may be involved in the regulation, clearly another gene product must be involved.

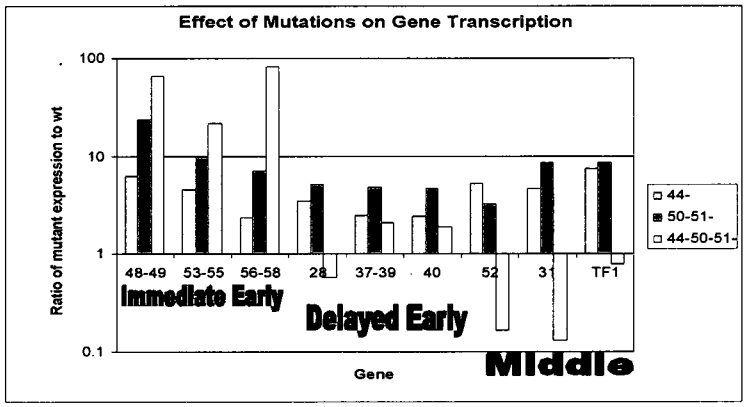

Figure 1.2: Effect of Mutations on Specific Gene Transcription: RNA was harvested 15 minutes post infection for wt, 44-, 50-/51- and 44-/50-/51- SPO1 strains. The RNA was labeled and hybridized to dot blots on a positively charged nylon membrane, which was visualized on film. Amount of RNA was determined by densitometer. Relative amounts of RNA were determined by dividing the densitometer reading for each gene in an experimental strain by the reading for the gene in the wt strain. Genes are arranged based roughly on when their peak transcription occurred (data from Sampath and Stewart, 2004).

While the individual knockout mutants show a negative regulatory role for gp 44 and 51, the triple mutant 44^{-} $50^{-}51^{-}$ indicates a potential stimulatory role for delayed early and middle gene expression. The triple mutant shows a decrease in expression of delayed early genes compared to the individual mutants and in some cases, a decrease compared to wild type. All middle genes tested showed an even more extreme decrease, with expression levels lower than wild type. This indicates that gp44 in the absence of gp51/50 and gp51/50 in the absence of gp44 can stimulate delayed early and middle early gene expression (Sampath and Stewart, 2004).

A potential mechanism for gps44 and 51's regulatory effect is suggested by mutational analysis, binding assays and sequence homology. Gp44 is believed to bind to RNAP. When gp 44 is expressed in uninfected cells, the effect is lethal (Wei and Stewart, 1993). However, cells with a mutation in RNAP are protected from this lethal effect (Wei and Stewart, 1995). This strongly suggests that RNAP and gp44 are binding partners. Furthermore, gp44 has been shown to bind to RNAP in in vitro studies (Severinov, 2006). Gps44 and 51 also include acidic hydrophobic domains which are similar to the domain required for E. coli σ54 to bind to RNAP (Tintut, et al, 1994). Genes 44 and 51 are expected to have similar functions as they share significant homology with each other (Stewart et al, 1998). The 44 and 51 mutants also share similar phenotypes, which strengthens the argument that both genes have the same target, RNAP.

1.7 Gene 25.1

Gene 25.1 may be involved in the regulation between immediate and delayed early genes. It has a putative early promoter and shares 33% and 34% identity with gps44 and 51 respectively (Stewart et al, 2009). As discussed above, gps44 and 51 are known to be transcription regulators.

More specifically, it has been shown that gps44 and 51, in the absence of each other, can stimulate delayed early and middle gene expression. With the high homology, it is possible that gp25.1 may also act in a similar positive regulatory manner.

A second potential activity for gene 25.1 is indicated by the results for the $44^{-}/50^{-}/51^{-}$ triple mutant. The triple mutant shows higher expression of immediate early genes than wild type or either single or double mutant strain (Sampath and Stewart, 2004). This level is higher then expected for just the lack of a repressor. It is possible that gene 25.1 stimulates immediate early gene expression when genes 44 and 51 are not available. Based on homology, gps 25.1, 44 and 51 may all be able to bind with RNAP, so they may compete for binding. One potential mechanism could be that when gp25.1 binds to RNAP, it could cause immediate early gene expression, whereas when 44 and 51 are binding to RNAP, immediate early expression is repressed.

1.8 Gene 2.21

An additional sigma factor could also explain the regulation of delayed early genes. Gp2.21 is extremely interesting as it appears to be a sigma

factor. It shares 24% homology with σK in B. subtilis and 31% homology with Clostridium perfringens' σK (Stewart et al, 2009). σK is expressed in B. subtilis only during sporulation. As SPO1 can only effectively infect B. subtilis during the exponential phase, there is no concern that the B. subtilis σK would affect the infection and takeover process. Delayed early genes, in addition to σA promoters, have promoters that are similar to σK promoters. If gene 2.21 is indeed an active sigma factor, which recognizes the σK putative promoter, this could explain the difference in timing between the delayed and immediate early gene expression. Immediate genes could be expressed using σA, while the delayed early are expressed using gp2.21.

Chapter 2: Materials and Methods

2.1 Bacterial and Phage Strains

Table 2.1 lists the bacterial and bacteriophage strains used in this work.

2.2 Media and Growth Conditions

LB, Penassay, VY (Difco) or NY (Difco) were used for growing cells in liquid. Cells were grown on plates made from 40 g/L Trypticase Soy Agar (TSA). Tryptose blood agar base (TBAB), at a concentration of 15.4 g/L or TSA, at a concentration of 12 g/L was used as top agar. Defined liquid media used were Spizizen's salts medium (SM) (Spizizen, 1958), C1 (SM plus glucose plus complete amino acids)(Stewart, 1969), C4 (C1 without leucine, isoleucine and valine, for *E. coli* 0.0204 mg/ml thiamine), C2 (SM plus 5% Casein Hydrolysate, glucose and 0.16 mg/ml Aromatic Amino Acids), and C3 (SM plus 4% glycerol, 0.4% glucose). Ampicillin (Amp), kanamycin (km) and carbenicillin were added to media at 50 µg/ml. Chloramphenicol (cm) was added to the media for *B. subtilis* at 5 µg/ml and 10 µg/ml for *E. coli*. Isopropyl B-D-thiogalactopyranoside (IPTG) stock solution was prepared at 100 mM.

Table 2.1 Bacterial and Bacteriophage Strains		
Strain	**Relevant Genotype or Description**	**Reference or Source**
Bacteria		
B. subtilis		
CB-10	his-trp-sup-	Glassberg et al, 1977
CB-313	his-met-sup3+	Glassberg et al, 1977
E. coli		
DH5	hsdR17 recA1 endA1 gyrA96 thi-1 relA1	Sambrook et al, 1989
Top Ten	Maintenance strain for pET101	Provided by Invitrogen Life Technologies
BL21 STAR	Contains IPTG inducible T7RNAP	Provided by Invitrogen Life Technologies
Rosetta 2 cells	Expresses codons not typically expressed in *E.coli*	Rosetta 2(DE3) Competent Cells for enhanced coverage of codon bias in E. coli (2003) inNovations 18, 28., provided by Novagen
Bacteriophage		
SPO1	wild type version	
SPO1 37-	nonsense mutation in gene 37	LL
SPO1 38-	nonsense mutation in gene 38	Laura Laughlin (LL) Stewart lab
SPO1 39-	nonsense mutation in gene 39	LL
SPO1 40-	nonsense mutation in gene 40	Bati Myles (BM) Stewart lab
SPO1 39-40-	nonsense mutation in genes 39 and 40	LL
SPO1 38-39-	nonsense mutation in genes 38 and 39	LL
SPO1 44-50-51-	nonsense mutation in genes 44, 50 and 51	Aruna Sampath (AS) Stewart lab
SPO1 40-44-50-51	nonsense mutation in genes 40, 44, 50 and 51	this work

Table 2.1 Bacterial and Bacteriophage Strains: The table shows the different strains of bacteria and bacteriophage that were used throughout this work, as well as a brief description of them and the relevant reference. For strains created in the Stewart lab, the name of the creator is instead referenced.

In order to prepare SPO1 lysates, a mid log phase *B. subtilis* culture was infected. The infected culture was shaken vigorously at 37°C until lysis occurred. The culture was then centrifuged in Sorvall RC-5B Centrifuge for 5 minutes at 5,000 rpm to remove cellular debris.

2.3 Oligonucleotide primers and probes

The oligonucleotides used in this work are defined in Table 2.2. Oligonucelotides were designed using the Amplify program and synthesized by Integrated DNA Technologies or Sigma Aldrich.

2.4 Plasmids

Plasmids were created by inserting genes into vectors pPW19, pET101 or pET28b (Table 2.3). The gene fragments were created by PCR using either SPO1 or pBM1 (for genes 38-39) and PBM5 (for genes 37-39). For inserts cloned into pPW19, HindIII sites were added through PCR. HindIII was used to cut the fragment and pPW19, by incubating at 37°C for one hour. Because both sides of the insert have HindIII sites, the insert can

Table 2.2 Oligonucleotides				
Oligo name	**Sequence**	**Brief Description**	**Starting position**	**Company**
Primers				
p37UT	CACCGAACAAGG AGGGGTGTACAT GAGTG	upstream of gene 37	1804	IDTDNA
p37DT	GATTATCTCCTCC TCTTAATATACC	downstream of gene 37	1919	IDTDNA
p783	CAGTTAAAGCTTT TACTGGTTTAGTT CCATATTGATTCC	end terminal for gene 38, HindIII site	2474	IDTDNA
pu277	CAAGGTGTGGCA TAATGTGTGGAAT TGTGAGC	upstream of insert in pPW19	277 in pPW19	IDTDNA
p39UT	CACCCCGGCACC TTAATGAGGAAT	upstream of gene 39 start site, used for Topo cloning	2386	IDTDNA
p1559	CGCATAAAGCTTA TACCCCTTTCTTA GGCACTAGTGG	downstream of gene 39, HindIII site	3250	IDTDNA
39DTM	TACCCCTTTCTTA GGCACTAGTGGA G	downstream of 39 with end terminal tag removed to allow his tag expression	3250	IDTDNA
p28B/39F	ATCGAGCCATATG ATGGAACTAAACC TAGA	upstream of gene 39 for cloning into pET28B, NdeI site	2482	IDTDNA
P28b/39R	AGACTGAAGCTTT TATACCCCTTTCT TAGGCACATTT	downstream of gene 39 for cloning into pET28B, HindIII site	3250	IDTDNA

p40DT	GGGTATCAGTCTT CAGTTTGCTCTAC	downstream of gene 40	4309	IDTDNA
p40UT	CACCCTCCACTAG TGCCTAAGAAAG GGG	upstream of gene 40	3223	IDTDNA
40DTM	GTCTTCAGTTTGC TCTACTCGCTTTT CTATGTCC	downstream of 40 with end terminal tag removed to allow his tag expression	4301	IDTDNA
6070	CAGTTAAAGCTTG TTCTCCTCATAAG	downstream of 2.21, HindIII site	26070	IDTDNA
5332	ACGTAGAAGCTTG ATGGGACGAGGA GTGGGG	upstream of 2.21, HindIII site	25332	IDTDNA
5009	ACG TAG AAG CTT GGT GGG AAG CGG GCT AAG AAA CTA GGT C	upstream of 2.21, HindIII site	25009	IDTDNA
8292	ACA CTC AAG CTT TGT AAT AAT TTG TTC AGG GCA AGA GG	upstream of 25.1, HindIII site	88292	IDTDNA
8990	ACA CTC AAG CTT CCG CAG TTC CCC CAT GTT GCT TGT C	downstream of 25.1, HindIII site	88990	IDTDNA
8264	ACG TAG AAG CTT TTT TCT GTG GAA TGG GGC AAA ATG ACT TCA ACA G	upstream of 25.1, HindIII site	88264	IDTDNA

2.21TAGmut	GGT CTA TAT TTA CAC TAG ATG AGT GTA AAA AGG GAT GAA GG	primer used to mutate 2.21wtA	25378	IDTDNA
2.21TAGmutRC	CCT TCA TCC CTT TTT ACA CTC ATC TAG TGT AAA TAT AGA CC	primer used to mutate 2.21wtA	25418	IDTDNA
2.21TAA	GAT GGG ACG AGG AGT GGG GAA AAT GTC TCA AAC TAA ATA AGA TGT AGG TCT ATA TTT A CAC	used to mutate p2.21wtB	25332	Sigma Aldrich-PAGE purified
2.21TAARC	GTG TAA ATA TAG ACC TAC ATC TTA TTT AGT TTG AGA CAT TTT CCC CAC TCC TCG TCC CAT C	used to mutate p2.21wtB	25390	Sigma Aldrich-PAGE purified
25.1TAGmut	ATG ATG GCA TAT GAA GTA GAA GGA TAG GTA GTT AGT ACT CTT AAG GAA CTA	Used to mutate p25.1wtA	88353	IDTDNA
25.1TAGRC	TAG TTC CTT AAG AGT ACT AAC TAC CTA TCC TTC TAC TTC ATA TGC CAT CAT	Used to mutate p25.1wtA	88403	IDTDNA

25.1TAA	GAA AGA TCT AAG TGA GGG TGG TTA ATT CCA TGA TGT AGT GTC TTT GGT TGA TGC	Used to mutate p25.1wtB	88427	Sigma Aldrich-PAGE purified
25.1TAA RC	GCA TCA ACC AAA GAC ACT ACA TCA TGG AAT TAA CCA CCC TCA CTT AGA TCT TTC	Used to mutate p25.1wtB	88427	Sigma Aldrich-PAGE purified
Probes				
2.21(5393) mut	CACTCACTATGTG TAAATAT	probe used to detect p2.21wtA	25393	IDTDNA
2.21(5393) wt	CACTCATTTTGTG TAAATAT	probe used to detect p2.21mutA	25393	IDTDNA
2.21(5360) mut	CAA ACT AAA TAA GAT GTA GGT C	probe used to detect 2.21mutB	25360	IDTDNA
2.21(5360) wt	CAA ACT AAA AAG GAT GTA GGT C	probe used to detect 2.21wtB	25360	IDTDNA
25.1(8377) mut	GAAGGATAGGTA GTTAGTAC	probe used to detect 25.1wtA	88377	IDTDNA
25.1(8377) wt	GAAGGAAAAGTA GTTAGTAC	probe used to detect 25.1mutA	88337	IDTDNA
25.1(8449) wt	GGG TGG TAA GTT CCA TG	probe used to detect 25.1mutB	88449	IDTDNA
25.1(8449) mut	GGG TGG TTA ATT CCA TG	probe used to detect 25.1wtB	88449	IDTDNA

LYS3UAG	AAATGGCTUAGTC AAATAA	probe used for 44-screening	5886	IDTDNA
LYS3AAA	AAATGGCTAAATC AAATAA	probe used for 44 wt screening	5886	IDTDNA
40mut	GGGGACTCAAAT ACG	probe used for 40-screening	3271	IDTDNA
40wt	GGGGACTCTAGT ACG	probe used for 40 wt screening	3271	IDTDNA

Table 2.2 Oligonucleotides: The oligonucleotides, used either as primers or probes throughout this work are defined. After the name of the primer, the sequence (5' to 3') and a brief description of the oligonucleotide are given. The location of the start site of the sequence in SPO1, or a plasmid where noted, is given.

Table 2.3 Plasmids

Plasmid	Vector	Insert	Reference/ Creator	Primers used	PCR template
pET28B			Novagen		
pET101			Studier, et al, 1990; obtained from Invitrogen Life Technologies		
pPW19			Wei and Stewart, 1993		
pET101lacZ	pET101	lacZ gene, used as control	Invitrogen		
pBM1	pPW19	genes 38-40 in expressed orientation, gene 40 is interrupted by insertion	BM		
pBM5	pPW19	37-39 in unexpressed orientation	BM		
pAZ51	pET101	51 with c-terminal his tag	Antonin Zwiefka (AZ), Stewart lab		
pPW19lacZ	pPW19	pPW19 with lacZ gene- used as control	Maurie Mintz (MM), Stewart lab		
pTY37	pET101	37 in expressed direction	TY	p37UT, p37DT	pBM5
pTY38F	pPW19	38 in expressed direction	Tameson Yip (TY) Stewart lab	p783, pU276	pBM1
pTY38R	pPW19	38 in unexpressed	TY	p783, pU277	pBM5
pTY39	pET101	39 expressed, no his tag	TY	p39UT, p1559	pBM1

pTY39C	pET101	39 in expressed direction with his tag added to c-terminal end	TY	p39UT, 39DTM	pBM5
pTY39N	pET28B	pET28B with gene 39 inserted with N-terminal his tag	TY	p28B/39F, p28B39R	pBM1
pTY40	pET101	gene 40 in expressed direction	TY	p40UT, p40DT	wt SPO1
TY40his	pET101	40 in expressed direction with his tag added to c-terminal end	TY	p40UT, 40DTM	wt SPO1
p25.1wtA	pPW19	25.1 in expressed orientation	TY	8292, 8990	SPO1
p25.1mutA	pPW19	25.1 mutated in expressed orientation	TY		
p25.1wtB	pPW19	25.1 starting after -35 site, in unexpressed orientation	TY	8264, 8990	SPO1
p25.1mutB	pPW19	mutated 25.1 starting after -35 site, in unexpressed orientation	TY		
p2.21wtA	pPW19	2.21 in unexpressed orientation	TY	5332, 6070	SPO1
p2.21mutA	pPW19	2.21 mutated in unexpressed orientation	TY		

p2.21wtB	pPW19	mutated 2.21 with 345 upstream bases in unexpressed orientation	TY	5009, 6070	SPO1
p2.21mutB	pPW19	2.21 with 345 upstream bases in unexpressed orientation	TY		

Table 2.3 Plasmids: This table outlines the plasmids used throughout this work. References are given for plasmids created outside the lab. Plasmids created by the lab are listed with the creator. The vector refers to which vector the insert was cloned into. For plasmids I created, the primers used for amplifying DNA for the insert, as well as the PCR template, are also listed.

clone into the vector in either direction. When the gene is cloned so that its expression is under control of the vector's promoter, the insert is considered to be in the expressed direction. When the gene is in the opposite direction, it is considered to be in the unexpressed orientation. For pET28b, a HindIII site downstream and an NdeI site upstream were added through the PCR primers. NdeI and HindIII plus buffer II [New England Biolabs (NEB)] were then added to the PCR fragment and the template and incubated for one hour at 37°C. The fragment was then ligated to pPW19 or pET28b by incubating the digested plasmid, the digested fragment and T4 DNA ligase (Roche) overnight at 16°C or at 37°C for 3 hours. The ligase reaction was deactivated by heating to 65°C for ten minutes. Ligation mixture was stored at 4°C until transformation, which was performed within 24 hours. pET101 was purchased from Invitrogen and uses the Topo system to incorporate a PCR insert (Invitrogen). A 6X C-terminal his tag can be added to a gene product by removing the stop codon with mutated primers. Plasmids were purified using Qiagen or MoBio kits, as described by the manufacturers. Table 2.3 shows the plasmids used throughout this work, with the insert and the primers used to create them.

2.5 DNA Transformation

DH5 and Rosetta2 cells were made competent through chemical means. Cells were grown overnight at 37°C and then diluted 1:100. The culture was allowed to grow to Klett 25-30 and then the cells were chilled on ice for 10 minutes. Cells were centrifuged for ten minutes at 5000 rpm at 4°C. The supernatant was discarded and the pellet resuspended in 25 mls of 50 mM $CaCl_2$. After incubating on ice for one hour, the cells were centrifuged for ten minutes at 3000 rpm at 4°C. The supernatant was again discarded and the pellet was resuspended in 3 ml of 50 mM $CaCl_2$/20% glycerol. The cells were either used immediately or stored immediately at -80°C. Top Ten and BL21 Star competent cells were purchased from Invitrogen.

Lab prepared competent cells were transformed by adding 5 µls of DNA to 200 µls of competent cells. Tubes of competent cells were incubated on ice for 30 minutes and then heat shocked at 42°C for 45 seconds. They were then immediately put in ice for 2 minutes. 1 ml of LB was added and the cells were incubated for 1 hour at 37°C. The cells were then plated and plates were grown overnight at 37°C. Top Ten and BL21 Star cells were transformed according to manufacturer's protocols (Invitrogen).

B. subtilis cells are naturally competent under starvation conditions. In order to transform them, an overnight culture was grown in 10 mls of Penassay with 0.1 ml 40% glucose, using a cotton plug. These were grown for 12-13 hours in a 37°C water bath with slow shaking. The cells were centrifuged for 5 minutes at 5000 rpm and then resuspended in 0.1 volume of SM with Amino Acid mix. 0.2 mls of the resuspended culture were diluted into 20 mls of C-1. Cells were grown until the culture was out of the exponential phase. This was determined by plotting the Klett reading versus time on a semi log graph. When the growth begins to level off, the cells are leaving exponential phase. The culture was then added to 80 mls of C2 and grown at 30°C with very slow shaking for 2 hours. For every 100 mls of competent cells, 1 ml of 2M $MgCl_2$ and 1 ml of 40% glucose were added. To transform the now competent cells, 1.0 µg of DNA was added to 1.0 mls of competent cells. Cells were grown at 37°C with normal shaking for 2 hours and plated on antibiotic plates for selection.

2.6 Polymerase Chain Reaction

When using SPO1 as a template, Taq polymerase (Eppendorf) was used: for all other reactions, Pfu (Stratagene) was used. dNTPs (Stratagene) were used at 0.2 mM concentration. All reactions were performed in a MJ Research MiniCycler. 0.05µg/µl of each primer was added. For SPO1 templates, the following program was used:

A: 94°C for 2 minutes.
B: 5 cycles of 3 segments each:
    94°C for 30 seconds
    44°C for 30 seconds
    72°C for 2 minutes
C: 30 cycles of 3 segments each
    94°C for 30 seconds
    50°C for 30 seconds
    72°C for 2 minutes
D: 72°C for 10 minutes
E: 15°C forever

For thymidine templates, the following program was used:

A: 94°C for 2 minutes.
B: 30 cycles of 3 segments each
    94°C for 30 seconds
    50°C for 30 seconds
    72°C for 2 minutes
C: 72°C for 10 minutes
D: 15°C forever

When the program states forever, that indicates that the program will hold the temperature until the user provides further input.

2.7 DNA, RNA and Protein Synthesis Assays

Macromolecular synthesis can be measured by pulse labeling. In order to label the synthesized macromolecules, for protein synthesis 4.0 µCi/ml H3-leucine (MP Biomedicals) was used, for RNA, 4.0 µCi/ml 5 H3-uridine (MP Biomedicals) was used, and for host DNA 4.0 µCi/ml H3-thymidine (MP Biomedicals) was used. Because SPO1 uses hydroxymethyl uracil instead of thymidine, this allows for testing phage and DNA synthesis separately. For RNA synthesis, during SPO1 infection, for the first 25 minutes of infection, the bulk of RNA synthesized is host RNA. Cultures were grown in C4 medium, which does not include leucine or DNA precursors. This encourages incorporation of the radioactively labeled precursors into the newly synthesized molecules.

When looking at the effect of expression of a gene on a bacterial cell, a bacteria culture containing the plasmid that expresses the specified gene was grown to the desired concentration. Experiments were performed with expression beginning at Klett 50, 15 or 1.5. For experiments where the culture was induced at Klett 1.5, the cultures were grown to Klett 15 (early exponential phase) and then diluted 1:10. Gene expression was

induced using 1 mM IPTG and unless otherwise stated, cells were grown at 37°C with shaking at 100 rpm. Figure 2.1 shows an overview of a single pulse measuring DNA synthesis. At varying times, an aliquot of 0.5

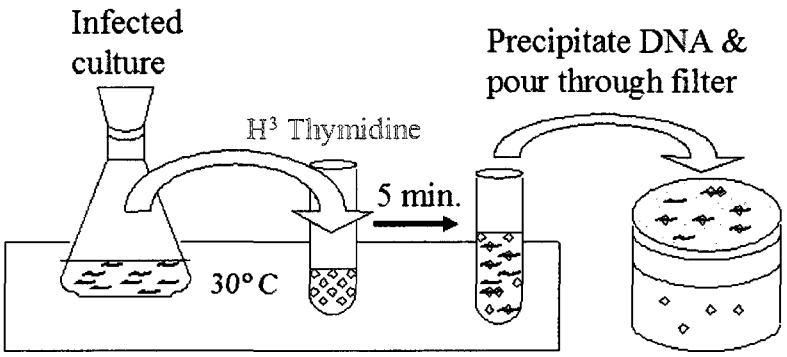

Figure 2.1 DNA Pulse: A cartoon representation of a DNA pulse is shown. An aliquot of the induced or infected culture is added to radioactively labeled DNA precursor. Newly synthesized DNA will include the radioactive precursor. The solution is then precipitated and poured over a filter. The DNA does not pass through the filter, but the unincorporated radioactive precursor does. The filter is then measured for radioactivity, which represents how much DNA synthesis occurred in the 5 minute pulse. Figure created by Laura Laughlin.

mls of the culture was added to 0.1 ml of the appropriate radioactive precursor. Incorporation of the precursor was allowed to proceed for five minutes and then the macromolecule was precipitated by adding 10% TCA. For the precipitates measuring protein synthesis, the precipitate was first boiled for 15 minutes and then cooled on ice for a minimum of 10 minutes. For all others, the precipitate was stored briefly on ice, for no

more then two hours. The precipitate was then run through a 0.45 micron nitrocellulose filter that collects the radioactive macromolecule, while allowing the unincorporated radioactive molecule to flow through. The filters were individually washed with 30 mls of cold 0.3% TCA in DSC (0.015 M NaCl, 0.0015 M NaCitrate). The radioactivity of the filter was then measured, determining the rate of synthesis. Measuring was performed by placing the dried filter into a filmware bag with 1 ml of scintillation fluid and then counting using a scintillation counter.

In order to determine the phenotype of the nonsense mutants, a similar process was followed. CB10 cells were grown to mid- exponential phase and then the culture split into smaller aliquots. This allows for comparison between wild type and mutant strains with an essentially identical starting point. The CB10 cells were infected with $5x10^8$ phage/ml, leading to a multiplicity of infection (MOI) of approximately 5. Five minute pulses were performed at varying times after expression. Cultures were also plated to determine the MOI immediately prior to infection and 5 minutes post infection.

2.8 Colony Formation Assay

Bacteria containing plasmids expressing SPO1 genes were grown to mid exponential phase. The genes were then induced by adding 1 mM IPTG. At different times after gene induction, samples of the colony were plated on antibiotic TSA plates in duplicate. The colonies on each plate were counted and the two plates averaged. In order to insure that plates with a low enough concentration of colonies were obtained, the colonies were plated at multiple dilutions. When plates from multiple dilutions were countable, the lowest dilution plates were used.

2.9 Isolation of gp38-Resistant and gp39-Resistant E. coli strains

Expression of genes 38 and 39 led to deficient growth. Bacteria containing either pTY38F or pTY39 were plated on antibiotic TSA plates containing IPTG. The plates were grown overnight at 37°C. Colonies that were capable of normal growth were selected as potential mutants. In order to determine if the mutations were in the plasmid, the plasmid was purified with the Qiagen rapid plasmid prep, following the manufacturer's protocol. The purified plasmids were transformed into wild type cells and plated on plates with and without IPTG. If the lethal effect was still

present, it was assumed that the mutation was within the cell's chromosome. If the plasmid had mutated, then the cells would grow equally well on plates with or without IPTG. If the mutation was chromosomal, the cells were then cured of the plasmid by growing a culture at 37°C, with normal shaking, diluting as necessary, for several days. In order to determine if the cells were cured, the culture was plated on plates without antibiotics and grown overnight. Individual colonies were then streaked onto plates with and without the appropriate antibiotic. When the colony has lost its plasmid, it will no longer be resistant to the antibiotic. If the streak did not grow on the antibiotic plate, but did grow on the plate with no antibiotic, that colony was considered cured.

Since the pET101 expression system requires expression of T7 RNAP from the cell's genome, it is possible that a cellular mutation would disable expression of T7 RNAP. This would disable expression of any gene from the plasmid and would not be a useful mutation to isolate. To test for chromosomal mutations in T7 RNAP, the cured bacterial culture was then transformed with pAZ51, a plasmid expressing gene 51, which was previously shown to be highly lethal. The transformed cells were grown on plates with IPTG. If the culture was resistant to the effects of this gene, it was assumed that the mutation was not specific to gene expression. A

plasmid expressing lacZ was also used to screen for mutations which disabling gene expression from the plasmid. These cultures were plated on plates containing X-gal. Colonies unable to express the gene would be white (instead of blue), indicating that the colony was not resistant to the expressed gene. None of our potential resistant mutants passed all tests.

2.10 Plaque and Colony Lifts

DNA can be lifted from TSA plates containing either plaques or bacterial colonies. Plaques or colonies were lifted from the plates onto S&S BA85 Protran membranes (Schleich and Shuell). Membranes were gently laid directly onto the plates and removed when the full surface area was wet. These filters were then exposed to 0.5 N NaOH/1.5 M NaCl by placing the filter onto 3MM paper saturated with the solution. After 5 minutes, the filter was moved to 3MM paper saturated with 1.0 M Tris pH 7.4 and then moved to 3MM paper saturated with 0.5 M Tris pH 7.4. The DNA was then UV crosslinked to the membrane using the Stratagene UV Stratalinker.

2.11 Isolation of 40⁻44⁻50⁻51⁻SPO1 Mutation

A culture of *B. subtilis* CB313, a suppressor strain, containing the plasmid pJT3 (pPW19 with mutated gene 40) was infected with 44^{-}50^{-}51^{-}SPO1 (created by Aruna Sampath) at a MOI of one. When phage infects the CB313 with the plasmid, the phage DNA and the plasmid are capable of homologous recombination, effectively transferring the mutation from the plasmid to the phage. Fifteen minutes after infection, the infected cells were diluted 1:50. After 45 minutes, 0.2 mls of chloroform per 10 ml of infected culture was added to the diluted culture. The lysate was then plated for plaques by adding CB313 grown to mid exponential phase, and the ysate to15.4 g/L TBAB top agar. Plates were grown overnight at 37°C, then moved to room temperature for 8-24 hours. They were then stored at 4°C for 1-4 days and plaques lifted as described in Ch 2.10.

Oligos 40wt15 and 40mt15 were labeled with flourescein 11-dUTP. 5.32 µls Cacodylate buffer, 3.325 µls Flourescein 11-dUTP, 1.7 µls of 0.1 µg/µl oligo in 10 mM Tris 7.4 buffer, 2.7 µl terminal tansferase and 40.2 µls of H_20 (Amersham Biosciences) were mixed in a microcentrifuge tube with a pipette tip. The solution was incubated for 90 minutes at 37°C. The labeled probes can be stored at -20°C for up to one month or used immediately.

Membranes were placed into the hybridization tubes with 6.6 mls of hybridization buffer (25.2 ml H2O, 30 ml CSC, 0.6 ml 10% SDS, 3 ml liquid buffer, 300 mg dextran sulfate MW 500000). The tubes were incubated in Robbins Scientific Model 400 Hybridization incubator for 60 minutes at 27°C, rotating at 4 rpm. For each membrane, 16 µls of labeled oligo were added to the tube. Tubes were rotated overnight. The hybridization solution was discarded and the membranes washed with 20 mls of 5X SSC/0.1% SDS. The membranes were moved to a glass pan and 536 ml of 5XSSC/ 0.1% SDS were added. After 5 minutes of gently shaking, the solution was removed. This wash step was repeated once. The membranes were then washed with Buffer 1 (17.5g NaCl, 4.72g Tris base, 25.4g Tris HCl, 2000 ml H_20) for 1 minute. For each membrane, 13.2 mls of 1:20 liquid block (Amersham Biosciences) was added and then incubated at room temperature on a rotary shaker at 100 rpm for 30 minutes. Membranes were rinsed with 636 ml buffer 1 for 1 minute. The buffer was then discarded and then, for each filter, 15 mls of antibody solution [5 mg/ml BSA with 1:100 antiflourescein HRP conjugate mixed in buffer 2 (46.8g NaCl, 4.72g Tris base, 2000 ml H_2O)] was added. The membranes were then incubated for 30 minutes on a rotary shaker at 100 rpm. Membranes were washed with 636 ml buffer 2 twice for 5 minutes.

To detect the signal, 3.5 mls of Detection solution 1 were mixed with 3.5 mls of Detection Solution 2 from the ECL Detection Reagents (Amersham Biosciences). 6.6 mls of mixed detection solution were distributed onto the membranes evenly. After one minute of incubation, the membranes were drained by touching the membrane to 3MM paper and then wrapped in Saran Wrap. Film was exposed to the wrapped membranes for a minimum of 9 hours and then developed.

Plaques that tested positive were picked and resuspended in 1 ml Penassay. The resuspended single plaque was then plated. These plates were tested in the same manner as in the original screening experiment. Since the mutation is in the terminal redundancy, an originally positive plaque will lead to plaques with 2 mutant copies of the gene, 1 mutant and 1 wild type copy of the gene or 2 wild type copies of the gene. For this reason, both a primary and a secondary lift were performed from the putative mutant plate and probed with either wild type or mutant probe. A plaque where the DNA hybridizes successfully to just the mutant probe and not the wild type probe was considered homozygous for the mutation. Sequencing was performed by Lonestar Labs to confirm the mutation.

Ch 3: Role of Genes 37-40

In order to understand the role of genes 37-40, two different approaches were used: nonsense mutants and gene expression in uninfected cells. Nonsense mutants were created by mutating a lysine codon early in the gene to a stop codon. Mutant phage lysates were then used to infect CB10 *B. subtilis* and the infections monitored. Pulse labeling, described in Ch 2.7, was used to monitor the biosynthesis of host DNA, total RNA, and total protein. By comparing the rate of macromolecular synthesis between the mutant strains and the wild type strain, the effect of the genes on host shutoff can be determined. For example, if shutoff of DNA synthesis is deficient in a mutant strain, then it is likely that the gene product plays a causative role in host DNA synthesis shutoff. If shutoff of DNA synthesis is accelerated in the mutant strain, then the gene probably acts to restrain host DNA shutoff.

The second approach to determining the effect of the gene products was to express the gene in uninfected bacterial cells. The gene was cloned into either pPW19 or pET101, which put the genes under control of an IPTG- inducible promoter. After gene expression is induced, colony formation assays (Ch 2.8) and pulse labeling (Ch 2.7) experiments are

performed. Since these genes are believed to be involved in the shutoff of host RNA, DNA or protein, it is expected that expression will be detrimental and most likely lethal to the cell's growth. This assumes that the gene products can bind to bacterial cell targets and carry out their role without other SPO1 genes or cofactors. Since gene expression did show a detrimental and, for genes 39 and 40, lethal effect, macromolecular synthesis, measured by using pulse labeling, was performed. It was hoped that these experiments would more precisely explain the role of the gene product. For example, if the gene product is involved in shutting off DNA synthesis, when the gene is expressed, the amount of DNA being produced should drop. The last experiment performed using the expression plasmids was a simple plating assay. The cultures were plated on inducing plates and grown at 37°C. This experiment provides the least specific information, as the lack of growth could indicate a biostatic effect or a lethal effect and it gives no quantitative data regarding the growth rate. However, it is useful to quickly determine if gene expression has any effect on the cells.

3.1 Gp 40 plays an essential and causative role for the normal shutoff of host RNA and DNA synthesis.

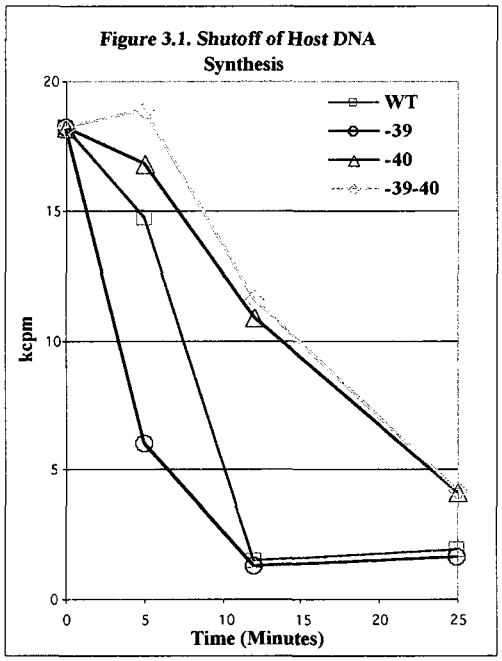

Figure 3.1: Shutoff of host DNA synthesis (39^-,40^-, 39^-40^-). Rates of DNA synthesis were measured, by pulse-labeling with [methyl-^{3}H] thymidine, as a function of time after infection by the indicated strains. The graph shows TCA-precipitable kcpm; i.e. cpm X 10^{-3}. Error shows a representative set of data and was reproduced numerous times. Data produced by Bati Myles.

Figures 3.1 and 3.2 clearly show that gene 40 knockout mutants are deficient in both RNA and host DNA synthesis shutoff (Bati Myles, Laura Laughlin data). When the wild type strain infected CB10, within 5 minutes host DNA shutoff had begun, as shown by the significant decrease in the rate of DNA synthesis. By 12 minutes shutoff was nearly complete. When the 40^- strain infects CB10, there was a small decrease in the rate of DNA synthesis after 5 minutes, but the amount of synthesis was still greater than wild type. By 12 minutes, the difference in shutoff was even more dramatic, with the 40^-

strain synthesizing more DNA than the wild type strain, throughout numerous repetitions of the experiment. At 25 minutes, the 40⁻ strain had significant shutoff, but still produced over twice as much DNA as the wild type strain. This indicates that some DNA shutoff can still occur without gp40; however, gp40 is essential for complete shutoff of host DNA to occur.

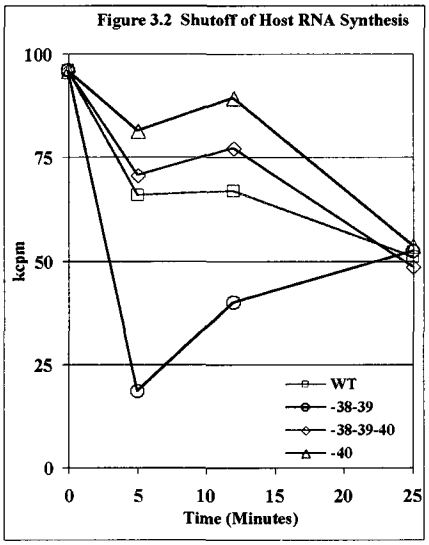

Figure 3.2: Shutoff of host RNA synthesis (40⁻, 38⁻39⁻, 38⁻39⁻40⁻). Rates of RNA synthesis were measured, by pulse-labeling with [5-^{3}H] uridine, as a function of time after infection by the indicated strains. The graph shows TCA-precipitable kcpm; i.e. cpm X 10^{-3}. Graph shows representative data. Data produced by Laura Laughlin.

For the shutoff of host RNA synthesis, by 24 minutes, RNA levels from 40⁻ infected cells were similar to those in wild type; however at 5 and 12 minutes, there was significantly more RNA synthesis in the 40⁻ strain. This indicates that gene 40 is essential and causative for the shutoff of RNA, but it is not the only gene involved in the process.

The dual function of gp40 (without genomic degradation) appears to be unique to SPO1. We do not believe that the shutoff of RNA synthesis leads to the shutoff of DNA synthesis or vice versa. While the timing of the experiments is not precise enough to say conclusively that one shutoff does not cause the other, the intervals between the time points are relatively short make this unlikely. It has been shown that when RNA synthesis is shutoff in *E.coli*, the resulting shutoff in DNA synthesis occurs very slowly, at rates where the causation would be clear under our experimental conditions (Lark, 1972). Furthermore, in other nonsense mutants, such as 39^{-} and $44^{-}50^{-}51^{-}$, RNA and DNA synthesis have different phenotypes. If the shutoffs were connected to each other, the mutants would have a similar phenotype for both types of shutoff, as the effect on one shutoff would cause a similar effect on the other shutoff.

3.2 *Gene 40 expression in uninfected cells causes varying degrees of lethality in E. coli.*

The gene 40 mutants indicate a causative role in host RNA and DNA synthesis shutoff. However, when gene 40 was expressed in uninfected cells, the effect varies greatly, depending on the conditions. Under several conditions, there was no difference in gene 40 expression and expression of a lacZ control (referred to as BG) (figure 3.3). When gene

40 was expressed in cells induced at Klett 1.5 and grown in C4 medium, a clear lethal effect was seen (figure 3.4). Under these conditions, the control strain barely grew, indicating that these were harsh conditions for this strain. Since the cells were already stressed, the addition of gp40's possibly minor toxic effect was

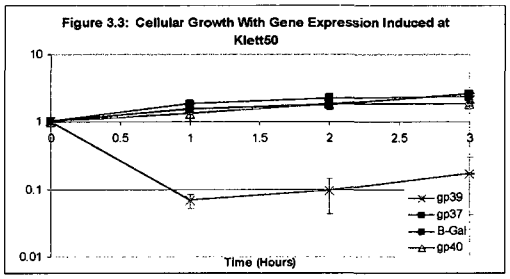

Figure 3.3: Cellular Growth With Gene Expression Induced at Klett50. Liquid cultures of BL21 STAR containing pTY37, pTY39, pTY40 or pET101lacZ, grown to Klett50 in LB media, were plated on non-inducing plates immediately before gene induction and then every hour for three hours. The number of colonies on each plate was counted and divided by the number of colonies on the initial plates. Numbers greater than one indicate continued growth. Numbers less than one indicate cell death. B-gal is a control containing pET101lacZ. Data shows representative data from 3 experiments.

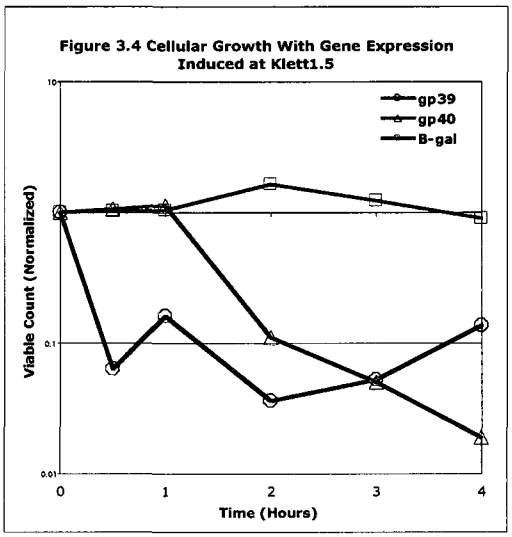

Figure 3.4: Cellular Growth With Gene Expression Induced at Klett1.5. Liquid cultures of pTY39, pTY40 or pET101lacZ, grown to Klett15 in C4 media and then diluted 1:10, were plated on non-inducing plates immediately before gene induction and then every hour for four hours. The number of colonies on each plate was counted and divided by the number of colonies on the initial plates. Numbers greater than one indicate continued growth. Numbers less than one indicate cell death. B-gal is a control containing pET101lacZ. Data shows representative data from 3 experiments.

overwhelming. There was a clear effect in this condition, which allowed for further experiments examining the effect on the rate of macromolecular synthesis after expression of the gene. Unfortunately, for RNA and DNA synthesis, the experiments varied greatly from one to another, making it difficult to interpret the results. For protein synthesis, a slow shutoff is observed, taking at least 2 hours for complete shutoff. Figure 3.5 shows the results from one representative experiment where the rate of protein synthesis was measured. Because of the slow change in rate occurring over two hours, this shutoff is not believed to be the primary lethal mechanism.

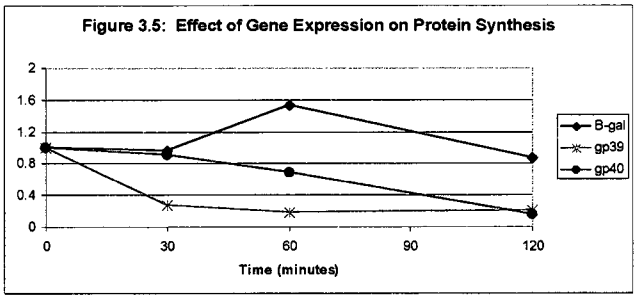

Figure 3.5: Effect of Gene Expression on Protein Synthesis Rates of protein synthesis were measured, by pulse-labeling with [methyl-^{3}H] leucine, as a function of time after induction of the BL21 STAR culture containing pTY39, pTY40 or pET101lacZ, grown to Klett 15 and then diluted 1:10. Results were normalized by dividing the cpm at each time point by the cpm at time 0. B-gal is a control containing pET101lacZ. Graph shows representative data from 3 experiments.

A potential explanation for the limited, conditional lethality of gene 40 expression could be explained by the use of *E.coli* in the experiments. *E.*

coli was used due to difficulties cloning the genes into pPW19. pPW19 is not as tightly regulated, so lethal genes are often impossible to clone using this vector. We wanted to compare the effects of expression of all four genes with each other, so once one gene proved impossible to clone in pPW19, all future genes were cloned in pET101. Since *E. coli* is not the native host, gp40 may not be able to effectively bind with the *E. coli* version of its target. It is also possible that the gene products require another SPO1 protein to function effectively. Since neither RNA nor DNA shutoff is completely deficient in the 40^- mutants, at least one other gene product must be involved in the host RNA and DNA shutoff. The lack of this other gene product(s) may limit the effectiveness of gp40's ability to shutoff host DNA and RNA synthesis.

3.3 Gene 39 regulates host DNA shutoff.

As seen in figure 3.1, a nonsense mutant of gene 39 shows accelerated shutoff of host DNA synthesis compared to wild type (Laughlin data) in numerous repetitions of the experiment. While the wild type strain is able to "catch up" with the 39^- strain by 12 minutes, at five minutes, a significantly higher amount of shutoff has occurred in the 39^- mutant. Since removal of gp39 allows host shutoff to occur more quickly, gp39

must act to inhibit the shutoff of DNA synthesis. This indicates that gene 39 likely plays a regulatory role in the host DNA shutoff process.

3.4 Gp38 and gp39 regulate host RNA shutoff.

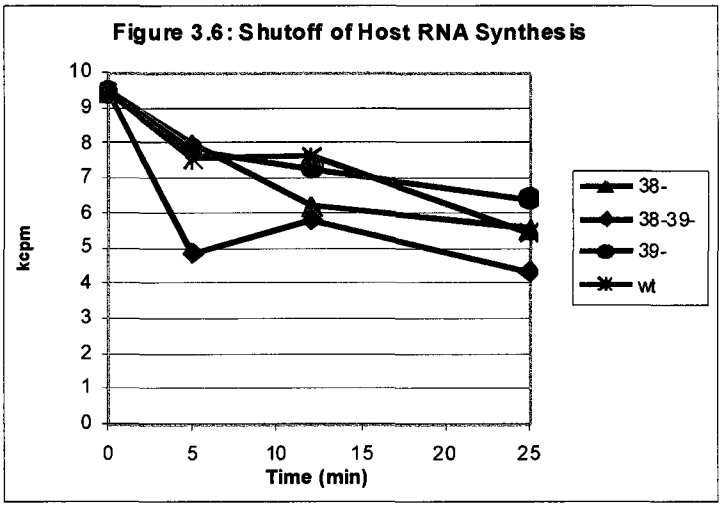

Figure 3.6: Shutoff of host RNA synthesis (38$^-$,39$^-$,38$^-$39$^-$). Rates of RNA synthesis were measured, by pulse-labeling with [5-^{3}H] uridine, as a function of time after infection by the indicated strains. The graph shows TCA-precipitable kcpm; i.e. cpm X 10^{-3}. Data is representative from several experiments. Data produced by Laura Laughlin.

Gene 38 and 39 mutants have a very limited RNA synthesis shutoff phenotype, but when both genes 38 and 39 were mutated (see figure 3.6 and 3.2, Laughlin data), there was acceleration in RNA shutoff, similar to the acceleration seen with host DNA shutoff in the 39 single mutant. This indicates that gps 38 and 39 must play a role in the regulation of RNA shutoff. Additionally, when either gp38 or 39 is not present, the other gene product must be able to compensate. Otherwise,

a greater effect would be seen in the single mutants. Gene 39, like gene 40, is implicated in both RNA and DNA synthesis shutoff, with gp39 acting in a negative regulatory role. The simplest, but not the only, explanation for this data is that gp39 acts to restrain gp40.

3.5 Gene 39 expression is lethal to uninfected bacterial cells.

Indications of gp39's lethal nature were apparent from the start of experiments. Our initial attempts to clone gene 39 into pPW19 were unsuccessful. While pPW19 was the preferred vector as it could be expressed from both *E. coli* and *B. subtilis*, pPW19 does not fully repress gene expression. If gp39 is sufficiently lethal, even minimal expression will kill any cell that contains the plasmid, making cloning the gene impossible. A plasmid with tighter gene regulation, pET101, was successfully used to clone gene 39 (pTY39). As expected, expression of gene 39 in uninfected cells did indeed have a definite lethal effect in every condition tested (figures 3.3, 3.4). This shows that gene 39 is capable on interacting with a cellular target, an interaction that leads to death. This was interesting as the 39^- phenotype indicates that gp39 plays a regulatory role, not a causative one.

When the rate of macromolecular synthesis was monitored after induction of gene 39, DNA synthesis rates varied greatly from one experiment to another, making it impossible to draw any conclusion regarding the effect of gp39 on DNA synthesis. In both of the experiments measuring RNA synthesis, by two hours, the rate of RNA synthesis was greater than at the time of gene induction. This indicates that gp39 does not shutoff RNA synthesis. However, in all three experiments examining protein synthesis, within 30 minutes (the first time point measured) a significant drop in protein synthesis was seen (figure 3.5) Since no corresponding drop in RNA synthesis was observed, it appears as though the gene product specifically affects protein synthesis. For example, after 30 minutes of expression, RNA was being synthesized at a lightly faster rate then before induction (1.09 and 1.37 times the initial amount). However, protein synthesis dropped steadily (0.27, 0.52 and 0.61 times the initial amount). The drop in protein synthesis could also be explained as a result of a separate primary lethal mechanism. However, the very early shutoff makes this less likely. Further experiments, looking at earlier time points, should be performed to clarify this unusual result.

3.6 Gene 38 expression has a detrimental effect on uninfected *E. coli* cells, but not *B. subtilis* cells.

While the 38$^-$ strain showed no significant phenotype when the rate of macromolecular synthesis was measured, colonies expressing gene 38, grown on inducing plates took nearly twice as long to grow as a culture containing pTY38R or pPW19lacZ grown on inducing plates. However, experiments monitoring the growth of a culture in liquid media (as defined in Ch 2.8) for three hours after induction of gene 38 were inconclusive, with each experiment giving different results. In the future, the experiments need to be repeated for more conclusive results.

Interestingly, when gene expression was induced in *B. subtilis*, there was no difference in growth on inducing plates and non-inducing plates. This result was surprising since the gene product should be able to interact with its native host more specifically than with *E. coli*. Since the result was so unexpected, we tested several different strains of *B. subtilis* to see if this was a universal *B. subtilis* response to the gene or if my strain was naturally resistant. CB313(pTY38F), CB307(pTY38F), CB324(pTY38F), CB325(pTY38F), CB312(pTY38F), CB332(pTY38F) and CB369(pTY38F) all showed no difference between colonies grown on inducing plates and

non inducing plates, showing that the lack of response to gp38 in *B. subtilis* cells is probably a universal *B. subtilis* response. Determining the gp38's binding partners may help explain why the gene product is lethal in *E. coli* but not *B. subtilis*.

3.7 Accelerated host-shutoff phenotypes caused by the 39⁻ mutant, the 38⁻39⁻double mutant, and the 44⁻50⁻51⁻triple mutant, are all suppressed by the 40⁻ mutation.

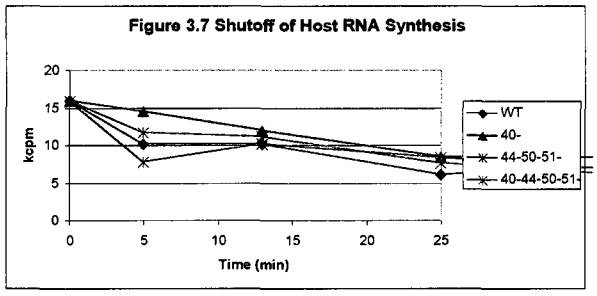

Figure 3.7: Shutoff of host RNA synthesis (40⁻, 44⁻50⁻51⁻, 40⁻44⁻50⁻51⁻). Rates of RNA synthesis were measured, by pulse-labeling with [5-^{3}H] uridine, as a function of time after infection by the indicated strains. The graph shows TCA-precipitable kcpm; i.e. cpm X 10^{-3}. Experiment has only been performed once.

Based on the phenotype of the 39 and 40 mutations, one hypothesis is that gp39 regulates gp40, while gp40 acts to shutoff RNA and DNA synthesis. If this is true, it is expected that a double mutant of 39⁻ and 40⁻ would have the same phenotype as the 40⁻ mutant. As seen in figure 3.1, for host DNA shutoff, this was indeed what happened. As seen in figure 3.2, when RNA

synthesis was assayed, the triple mutant 38$^-$39$^-$40$^-$ mostly suppressed the 38$^-$39$^-$ phenotype as well.

As discussed in the background and shown again in figure 3.7, the 44$^-$50$^-$ 51$^-$ mutation also has the accelerated host RNA shutoff phenotype. Further evidence discussed in Ch1.6 shows that the gps44 and 51 are regulatory proteins. We wanted to determine if these gene products were involved in regulating gp40. If these gene products do act on gp40, we expected that adding the 40$^-$ mutation would suppress the 44$^-$50$^-$51$^-$, as it did with the 39$^-$ mutation. In the one experiment testing this quadruple mutant, this suppression was observed (figure 3.7). In this experiment, the triple mutant did not show as great an acceleration in RNA synthesis shutoff as seen in most other experiments (compare figure 3.7 to figure 1.1), which makes the suppression effect less dramatic than expected. These experiments strengthen our argument that gp40 is a causative factor for host RNA and DNA shutoff and that gps44, 51, 38 and 39 regulate gp40.

3.8 Gene 37 expression has a detrimental effect on uninfected E. coli cells.

Despite gene 37's small size, we believe it still plays a role in host takeover. When *E.coli* containing pTY37 were grown on inducing plates for 48 hours at 37°C, the colonies appeared ragged and discolored (mottled) compared to cells grown on non-inducing media for the same amount of time. This difference in appearance after 48 hours of growth did not appear when DH5(pET101lacZ) cells were grown on inducing media. This indicates that gp37 is able to interact in some way with the cell. When an individual mottled colony grown on inducing media was selected and grown on antibiotic plates, both large and small colonies grew. The pattern was very similar to the satellite colonies seen when plating transformants, with a large colony surrounded by the small colonies. When we attempted to grow the small colonies on antibiotic plates, no growth was detected after 48 hours, indicating that these bacteria had lost their antibiotic resistance and therefore, their plasmids. The large colonies grew on antibiotic plates without any problems. The large colonies also were able to grow on inducing plates without any detrimental effect. The lack of the mottled effect indicates that these cells are now resistant to the gene. This suggests that the mottled effect may come from the presence of two genetically different bacteria- one wild type

lacking an antibiotic resistant plasmid and one mutated to be resistant to gene 37 expression. In the future, this observation may be useful for selecting resistant mutants.

While the plating experiments indicate that gp37 is an active gene capable of performing some function, experiments testing the rate of macromolecular synthesis with the nonsense mutants (performed by Laura Laughlin) show no phenotype. When uninfected E. coli BL21 STAR cells were induced and rates of growth and macromolecular synthesis measured in C4 media, there was no observable effect. With the 48 hour growth required to see a clear difference between growth on inducing and non-inducing mutants, it is not surprising that all experiments looking at effects at 30 minutes to 3 hours showed no effect.

3.9 Future experiments to test theoretical roles of 37-40

The 4 genes are expressed from one operon and are likely translationally coupled. This increases the likelihood that they are all involved in the same process. The phenotypes from the nonsense mutants give strong evidence that gp40 is involved in shutting off RNA and DNA synthesis. Gp39 appears to restrain both RNA and DNA synthesis shutoff, while

gp38 restrains regulation of RNA synthesis shutoff. The simplest explanation is that all 4 gene products are involved in both RNA and DNA shutoff, with gps38 and 39 acting to restrain gp40's action. Gp37 probably also acts in this process in some currently undefined way. It is possible that the gene products interact with just one target, such as a chromosomal protein, which is capable of effecting both RNA and DNA synthesis. It is also possible that the gene products have multiple targets, one shutting off host RNA synthesis and the other shutting off host DNA synthesis.

In order to clarify the roles of these genes, determining their binding partners is crucial. Knowing the binding partners would help answer the question of how they are shutting off RNA and DNA synthesis. It would also determine whether or not the gene products acted individually or formed a complex with each other. Attempts to purify the gene products, in order to perform binding experiments, are described in Ch4. Another useful experiment would be to repeat the lethality and macromolecular synthesis experiments using *B. subtilis.* This may give clearer results as the target will be the native target. This will require finding a vector for *B. subtilis* which can sufficiently repress gene expression.

Ch 4: Protein Purification Attempts of Gp39, Gp40 and Gp51

4.1 Gp39 Purification Attempts

In order to understand how gp39 functions, knowing what other gene products it binds to is critical. Pull down assays, co-immunoprecipitiaton and phage display assays would all be useful for obtaining this information. These experiments all require a purified protein. We focused most heavily on purifying gp39 because of its strong effect on the uninfected cells.

4.1.1 Gp39 Expression

Initial attempts to purify gp39 used the plasmid pTY39C, which is pET101 with gene 39 inserted. A 6X C-terminal his tag is added to the protein by eliminating the termination codon. In purifying a protein, high expression is necessary. The plasmid was expressed in BL21* cells. In order to optimize expression levels, cells were shaken at 37°C at 100 rpm. Expression was induced at Klett 50 with 1 mM IPTG in one of the following medias: LB, L Broth, SM, Penassay, NY, VY or SOC. Aliquots were taken at 1, 2 and 4 hours. An additional experiment compared shaking at 30°C, 37°C and 26°C. Maximum yield was seen when cells were grown in

Penassay for four hours after induction, at 37°C. Based on the strength of the gp39 band when visualized on a SDS-Page gel, this expression level was still not sufficient for protein purification.

Since pTY39C did not have strong expression, gene 39 was expressed from a different vector, pET28b. This new plasmid, pTY39N places an N-terminal his tag onto the protein. The cells were expressed in Rosetta2 cells. The Rosetta2 cells express codons not typically expressed in *E. coli*. If a protein requires a codon not commonly used in *E. coli*, the Rosetta2 cells would allow an adequate expression level. Based on experience with pET28b in Rosetta2 cells, optimal expression usually occurs when induced with 1 mM IPTG with a culture at Klett 75-85. After induction, the cells were grown overnight at 25°C in LB medium, while shaking at 100 rpm. This protocol yielded high levels of gp39.

4.1.2 Gp39 Solubility

Gp39 was found in the insoluble fraction of the cellular extract. Proteins must be soluble in order to be purified. They can be insoluble for many different reasons, such as misfolding or the formation of inclusion bodies. While this problem can be solved by denaturing the protein, this introduces an additional problem of refolding the protein properly. In an

attempt to produce a soluble, native protein, numerous conditions were attempted, using the protocol defined by Bondos and Bicknell (Bondos and Bicknell, 2003). After cells were lysed, following the protocol outlined in the Bondos paper, the cellular extract was resuspended in Binding Buffer (Novagen) with one of the following additions: 10 mM DTT, 100 mM NaCl, 0.2 M $MgCl_2$, 1 M urea, 1 M L-arg, 10% glycerol, 0.5% NP40, 10 mM EDTA, 1% TCA. After incubating for one hour at room temperature, the cellular extract was run over a Microcron concentrator with a 100 kDa molecular weight cut off (Fisher) which allowed only soluble proteins to pass through the filter. The solution was then run on a gel, with the amount of gp39 assessed visibly. Only the 10 mM DTT Binding Buffer solution showed any increase in gp39 solubility and this solubility increase was minimal. Since no conditions were found that allowed for high yield of native soluble protein, we denatured the cellular extract with 8 M urea.

4.1.3 Gp39 Purification and Final Product

After induction and overnight growth, proteins were extracted using the BugBuster kit (Novagen) following the company's protocols, with the addition of 8 M urea. The cellular extract was then run over a Nickel

column, following the Novagen protocol. The gp was eluted with Elute Buffer (Novagen) with 8 M urea (see figure 4.1).

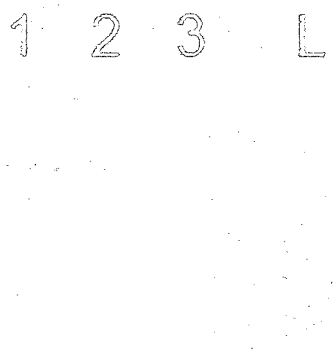

Figure 4.1 Gp39 Purification and Loss of his tag accessibility: 15 µl samples run on 8-16% SDS page gel (Bio-Rad). Lane one is purified gp39 in urea. Lane two is the soluble fraction of purified gp39 after dialysis and lane three is the dialyzed sample after being run over a nickel-chelate column. On the L (ladder) lane, the top purple band is 37.9 kd, with the next pink bad at 26.4kD.

In order to refold the protein, the cells were dialyzed to remove the urea using a slow step down process. The protein solution was placed in Snakeskin Pleated Dialysis Tubing 10,000 MWCO (Pierce), which was placed in Binding Buffer with a progressively lower dilution of urea. The solution was allowed to dialyze for 3 hours at 4°C at each dilution of urea. The 8 M urea was dialyzed to 6 M, then 5 M, then 4 M, then 3 M, then 2 M, then 1 M, then 0.5 M and then 0. At this point, we attempted to run the purified, refolded protein over a nickel column. Unfortunately, the protein did not bind to the column. When the purified protein was centrifuged, the protein was still found in the soluble portion based on gel visualization (see figure 4.1). This could indicate that

the his tag was folded into the protein so that it was no longer accessible. Since the his tag should be accessible, this suggests that the protein is not refolding properly. Testing binding properties with an unfolded protein would produce no useable data. Additionally, we planned to use the his tag in performing the pull down assays. Therefore, a new purification scheme must be determined. In future attempts, a tag on the C-terminal may refold properly, or a longer linker between the protein and the his tag may be successful. Alternately, a different tag, such as a maltose binding protein or a GST tag, could be used for purification.

4.2 Gp40 Purification

We desired a purified gp40 in order to perform the binding assay experiments discussed in section 4.1 for gp39. Gene 40 was inserted into pET101, adding a C-terminal his tag, to create pTY40C. The gp was expressed under the same conditions as pTY39C and like pTY39C, had problems with low yield and insolubility. Gp40 was tested with all the same conditions as gp39; however, none produced higher yield or greater solubility. Purification attempts did not proceed beyond this point.

4.3 Gp51 Purification Attempts

As discussed in Ch1. 6, one hypothesis we wanted to test was that gp51 and gp44 bind to RNAP to regulate gene expression. In order to test if Gp51 does indeed bind to RNAP, a pure protein was needed. In addition to testing if gp51 binds specifically to RNAP, pull down assays could be performed to determine currently unsuspected binding partners. Also, an in vitro transcription system could be devised, in order to look at the effect of gp51 on transcription. Different promoters could be used to determine the specific effect on early genes or delayed early genes. While expression and solubility of gp51 were not a problem, an additional complication arose in the form of an extra gene product (see Ch 4.4).

4.3.1 Gp51 Expression

Gene 51 was inserted into pET101, with the termination codon removed allowing for the inclusion of 6X C-terminal his tag (plasmid designated pAZ51). Gene expression was induced in BL21* cells, with 1 mM IPTG when the culture reached Klett 75-85. The cells were grown in LB medium for 2 hours at 37°C, shaking at 120 rpm. Additional conditions, such as temperature (room temperature and 30°C) and time after

induction (1, 2, 4 and 24 hours) were also tested. Two bands appeared with induction, one at the expected size and one around 10 kd. Figure 4.2, lane 3 shows an expressed culture, while lane 4 is pET101 with no insert, also induced with 1 mM IPTG. The two bands are indicated by black arrows. The top band is gp51, while the lower is an additional his tagged protein. This extra protein must be removed in order to obtain a pure protein, but the reason for its presence is worth studying at a later time.

4.3.2 Gp51 Purification

Induced cells were harvested using BugBuster (Novagen). The cell extracts were run over a nickel column, following manufacturer's protocol (Novagen). The protein was eluted with 1 M imidazole. Figure 4.2, lane one, shows this eluent. The additional protein fragment could be separated by running the cell extract over a DE52 (Whatman) column, as the smaller fragment runs through the column, while gp51 does not. 3.5 g of dry resin was resuspended in 30 mls 0.1 M Tris pH 8.0. The solution was allowed to settle for 15 minutes and then decanted. This was repeated until the pH was 7.5. The settled precipitant was then resuspended in 30 mls of Tris buffer (50 mM Tris pH 8.5, 0.5 mM EDTA). The solution was again allowed to settle and the supernatant decanted. The resin was then resuspended into 10 mls of Tris buffer. One ml of

resin was used for purification of protein from 1.5 grams of pelleted cells. Cellular extract was loaded onto the column and allowed to run through. The column was then washed with 32 mls of Tris buffer. The protein was eluted with 1.0 ml of Tris buffer with 1 M NaCl.

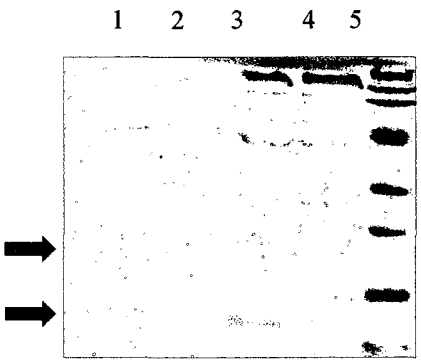

Figure 4.2 Purification of gp51: The upper arrow indicates the location of gp51, lower arrow indicates gp51 fragment. Lane 1 shows the result of an extract of cells expressing gp51 after being run over a nickel-chelate column. Lane 2 shows an extract of cells containing pET101 with no insert, run over a nickel-chelate column. Lanes 3 and 4 show the soluble fraction of cells expressing gp51 and cells containing an empty vector respectively. Lane 5 is the ladder. The lowest three bands are approximately 6.9, 21 and 29 kD.

While this column was capable of separating gp51 from the second protein, the eluent still contained contaminating proteins. It was hoped that by running the eluent over a nickel column, the remaining contaminants would be removed. Unfortunately, the elution buffer from the DE52 column does not react well with the nickel column. Alternatively, the Elution Buffer for the nickel column does not react well with the DE52 column. When the columns were run sequentially, it resulted in very low yields. Therefore, in the future, a dialysis step needs to be added between the two columns. Hopefully, this will fix the low yields and provide a pure protein.

4.4 Additional Gene Within Gene 51

The additional fragment that was expressed and co-purified with gp51 also contained a 6X C-terminal his tag, indicating that it is expressed from within gene 51. The fragment was submitted for N-terminal sequencing to UTMB Bimolecular Resource Facility following their outlined protocols. Their results indicated that the fragment was indeed expressed from the last third of gene 51. In addition, the same protocol was followed to confirm that the other band was indeed gp51. Our results clearly show high levels of expression (see figure 4.2), indicating that this gene product may play an important role in SPO1 infection. Since this gene was not known prior to these experiments and has such high expression, future studies looking at this gene are needed.

Ch 5: Gene 2.21 and 25.1 Mutant Screen

We believe genes 2.21 and 25.1 may be involved in the regulation of immediate early and delayed early genes (see Ch1). By studying mutants of these genes, we can examine the effects of the genes. For example, if the genes are the causative factor for delayed early gene expression, then the mutants will show a significant decrease in delayed early expression.

5.1 General Protocol

5.1.1 Homologous Recombination

In order to create a nonsense mutant, homologous recombination is used (see figure 5.1). When DNA is replicated, two sections of DNA that are homologous can line up, allowing for a crossing over event to occur. In our mutants, we create a plasmid containing a section of SPO1 DNA. A mutation can be introduced into the SPO1 insert, as described in later sections. If a reciprocal double crossover occurs between the SPO1 chromosome and the plasmid, one on each side of the mutation, the mutation would be swapped into the SPO1 genome. The plasmid would then have wild type SPO1 DNA instead of the mutated version it had originally carried. Homologous recombination is naturally occurring, so simply infecting bacteria containing a plasmid with SPO1 is sufficient.

However, it occurs at a relatively low frequency. In prior experiments, a successful recombination occurred only once in every thousand individual phage infections.

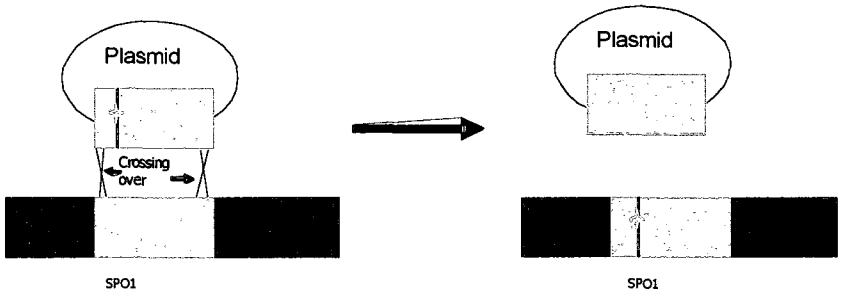

Figure 5.1 Homologous Recombination: In the figure, the light purple represents SPO1 DNA cloned into a plasmid. The yellow star represents an introduced mutation. The dark purple represents the SPO1 DNA not cloned in the plasmid. A crossing over event before and after the mutant site will transfer a mutation from the plasmid to SPO1. Increasing the homologous DNA before and after the mutation allows for more bases where crossing over can occur, increasing the frequency of mutation.

5.1.2 Mutant Plasmid Guidelines

In creating the mutant plasmid, there are several parameters to follow. Prior experiments, using genes found in the terminal redundancy, showed that homologous recombination occurred when 63 bases where present upstream and downstream of the mutation. A stop codon introduced at the beginning of the gene would essentially knockout the gene. Since this

mutation is potentially lethal to the phage, a *B. subtilis* suppressor strain, CB313 is used. In this suppressor strain, the TAA and likely also the TAG stop codon is read as a lysine. This means that if the first lysine in the gene is mutated then, in the suppressor strain, the gene can be translated as wild type (Mulbry et al, 1989). Once the mutant is created, the effect of the mutation can be studied by infecting a non suppressor strain (in our lab CB10) with the mutant strain. In order to make it easier to find the mutation, AAG is mutated to TAA or AAA to TAG. This change of two bases allows for easier differentiation between mutant and wild type when screening with an oligonucleotide probe. The base between the two mismatched bases, while not mutated, is still unable to bind to the wild type probe because of the mismatched pairs next to it. With 3 mismatched bases, the difference in binding rates between the mutant probe and mutant DNA versus the wild type probe and mutant DNA are different enough to effectively distinguish between mutant and wild type DNA.

5.1.3 Homologous Recombination Protocol and Initial Mutant Screen

In order to perform the recombination, *B. subtilis* CB313 cells containing a plasmid with the mutated gene were infected with wild type SPO1 at an MOI of one. Fifteen minutes after infection, the infected cells were diluted 1:50. After 45 minutes, 0.02 mls of chloroform per ml of infected culture

were added. The lysate was then plated for plaques, using CB313 grown to mid exponential phase, lysate and 15.4 g/L TBAB top agar. Plates were grown overnight at 37°C, then moved to room temperature for 8-24 hours. After that, they were stored at 4°C for 1-4 days. Plaque lifts were performed as described in Ch 2.10.

5.1.4 Hybridization

The membranes were then placed in hybridization tubes at 35°C, with 16 ml prehybridization solution [5X Denhardts (1g/100ml BSA, 1g/100ml Ficoll 400, 1g/100ml Polyvinyl Pyrolidine(sigma PVP-10)), 6X SSC, 0.5% SDS and 1 mM EDTA] for 4 hours in Robbins Scientific Model 400 Hybridization Incubator, rotating at 4 rpm at 35°C. While the membranes were incubating, labeled probes were prepared. While the radioactive probes can be used for up to a month after preparation, fresh probes are ideal as the radioactivity decays. 10 pm of primer was added to 10 µl of 10 mc/ml γ-^{32}P-ATP (MP Biomedicals), 4 µls of T4 Polynucleotide Kinase (PNK) (NEB) and 1X T4 PNK buffer, using H_20 to bring the volume up to 30 µls. The mixture was incubated for 45 minutes in a 37°C water bath. The enzyme was deactivated by a 20 minute incubation at 65°C and 40 µls of TE buffer added. The mixture was then run over a G-25/50 Sephadex column to purify it. 2.0 X 10^7 counts per minute of labeled probe were added to 26.4 mls of hybridization solution (5X Denhardts, 6X

SSC, 0.5% SDS). The prehybridization solution was removed and replaced with the probe and hybridization solution. The probes were allowed to hybridize overnight in a hybridization chamber set for 35°C, rotating at 4 rpm. After the overnight hybridization, the filters were washed twice for 5 minutes with 6X SSC at room temperature. They were then wrapped in saran wrap. Photographic film was exposed to the filters and the film developed.

5.1.5 Controls

In addition to the plaque lift membranes which were being screened, additional membranes were used as controls. One membrane was probed with a wild type specific probe. Since the wild type and mutant probes should have similar binding conditions and the probes were prepared in the same way at the same time, successful binding of the wild type probe to wild type plaque lifts would show that the probes were able to bind to the DNA under the conditions used. It also confirms that the plaque lift successfully picked up the DNA. While ideally, this would be done using mutant plaques, this is obviously not possible because if we had the mutant, we wouldn't need to do this experiment. While we could use the mutant plasmid in *E.coli*, this would be a very flawed control. When bacterial colony lifts are screened, they have a significantly stronger signal than plaque lifts. This could be due to differing levels of DNA being

transferred to the membrane. Based upon optimal exposure times for colony lifts versus plaque lifts, the signal is at least 30 times stronger. Therefore, we would expect a strong positive from a colony lift, even at times and conditions where we would not see a strong positive using a plaque lift.

A second control used was a blank membrane. This blank membrane shows that there is nothing else in the experiment causing a signal. Since we wanted to make sure that we saw any potential positive plaque, we often exposed the membranes long enough that the small amounts of non-binding radioactive probes were able to produce a signal. We were able to expose the films for a shorter time and still see a strong signal using wild type probes against wild type plaques with no signal on the blank membranes, but we wanted to make sure that no potential mutant was overlooked.

The final controls were the experimental plates probed with the mutant probe. Since we expected a low frequency of mutation, these plates were expected to be blank, with the exception of a few spots. This showed that the mutant probe did not bind strongly with wild type plaques. If we had seen most of the plaques, it would indicate that the probes were not

binding specifically enough to distinguish between mutant and plaque DNA.

5.1.6 Secondary Screening of Putative Mutants

After exposing the film and checking the controls, putative positive plaques were then picked. The plugs were resuspended in 1 ml of LB. These lysates were then plated so that each plate had approximately 200 plaques. Membranes were prepared and probed as described above. For any plaque that again produced a strong signal when probed with a mutant probe, the single plaque was picked and resuspended in 1 ml of LB. 5 µls of this lysate was amplified by PCR using the SPO1 protocols discussed in Ch 2.6. No additional preparation was needed to free the SPO1 DNA from the protein capsid for PCR amplification. It was assumed that the high temperature in the PCR protocol is enough to break the capsid and make the DNA accessible. This DNA was then sequenced by Lonestar or Seqwright sequencing companies.

5.1.7 Multiple Recombination Rounds

Unfortunately, following the above described protocol did not yield any mutants (more details discussed below). We therefore altered the recombination protocol in an attempt at increasing the frequency of mutation. CB313, containing the appropriate plasmid, was infected with the appropriate lysate, as described above. These cells were then

allowed to fully lyse. This lysate was then used to infect a new culture of CB313 (which also contained the appropriate plasmid). In theory, the lysate would have the original mutants, as well as newly formed mutants. In a perfect situation, the second lysate would have twice as many mutants as the original recombined lysate. Ten more rounds of this were performed. The lysate from the final round was then screened for mutant plaques.

5.2 Proof of Principle: Gene 44 Mutant

In order to show that the screening method works at distinguishing mutant phage from wild type phage, plates were prepared of plaques with a mutated gene 44, a wild type version of gene 44 and mixture of the two. As seen in figure 5.2, when the mutated plaques were probed with a mutant specific probe (LYS3UAG), strong spots were seen, but when the wild type probe (LYS3AAA) was used, no plaques were seen. For wild type plates, the wild type probe had a strong signal, whereas the mutant probe had no signal. On the mixed plates, half of the plaques were visible when probed with wt probe and the other half when probed with mutant probe. This shows that the detection system does work.

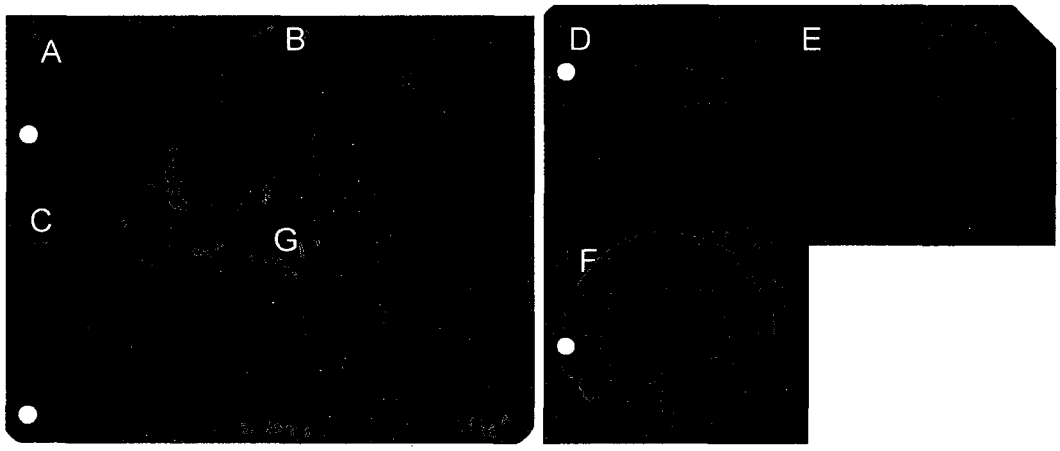

Figure 5.2 Gene 44 Mutant Detection: The film shows membranes probed with either the 44 mutant probe (LYS3UAG) or 44 wt probe (LYS3AAA). Membrane A is a mixture of wt and 44^- phage strains. Membrane B is 44^-. Membrane C is wt. Membrane D is a mixture of wt and 44^-. Membrane E is 44^-. Membrane F is wt. Membrane G is a blank membrane. Membranes A, B, C and G are probed with the radioactively labeled LYS3UAG, while membranes D, E and F are probed with the radioactively labeled LYS3AAA. The film was exposed to the membranes for 15 hours. Spots appearing on E, G and C are background contamination, perhaps caused by air bubbles in the filter.

5.3 p25.1mutA Recombination Attempt

An initial plasmid (p25.1wtA) consisting of the full 25.1 gene inserted in the expressed orientation into pPW19 (see Table 2.3 and figure 5.3) was

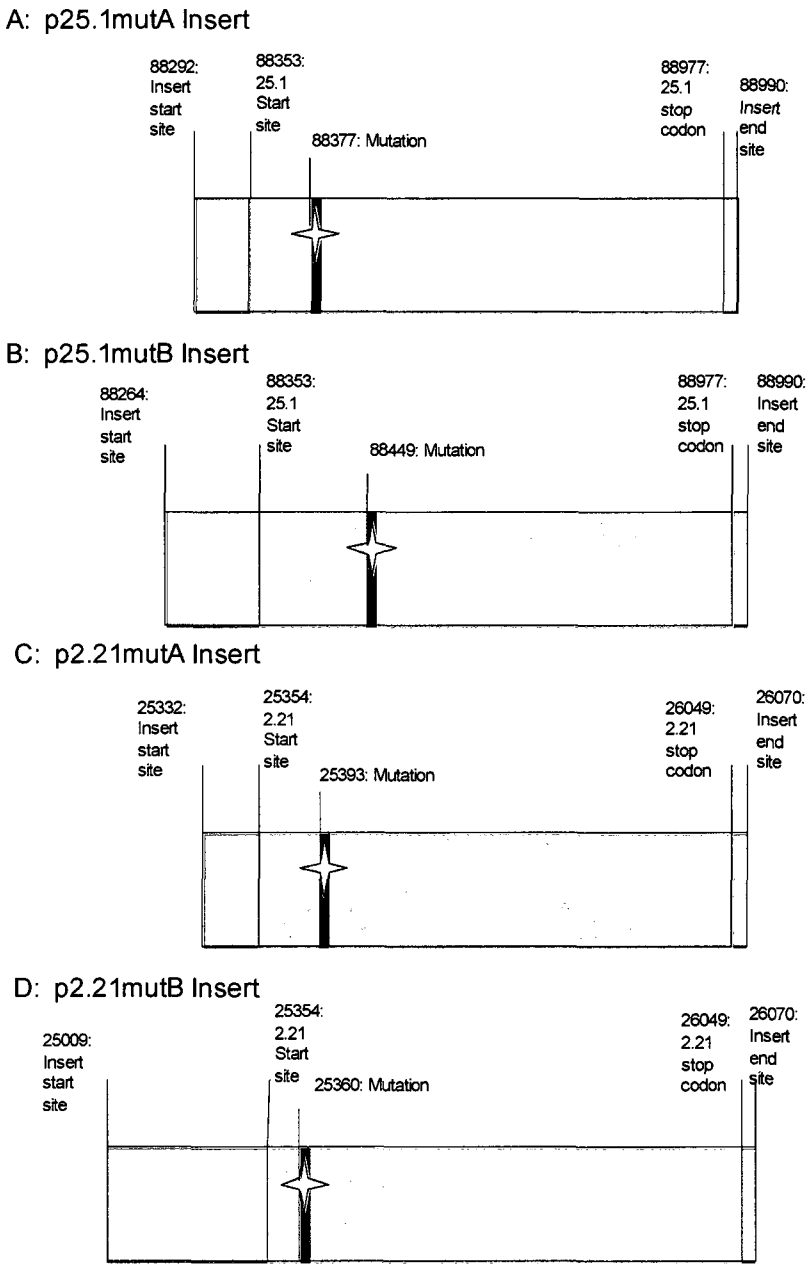

Figure 5.3 Plasmid Inserts: These cartoons compare the 4 mutated inserts created in this chapter: p25.1mtA (A), p25.1mtB (B), p.2.21mtA (C) and p2.21mtB (D). Diagrams are not to scale. The pink section represents the gene, while the purple sections are bases upstream and downstream of the gene. The blue star on a green background represents the introduced mutation.

mutated to create p25.1mutA. 61 bases upstream of the gene were also included. The entire promoter region was excluded. Since this gene is an early gene, including the promoter would allow for gene expression in bacteria. Since the two gene products that are homologous to 25.1 (44 and 51) were both very lethal, expression of this gene was likely also lethal. p25.1wtA was mutagenized using the Stratagene Quick Change Site-Directed Mutagenesis Kit, following the provided protocol. 25.1TAGmut and its reverse complement (25.1TAGRC) primers were used, creating a stop codon (TAG) instead of a lysine (AAA) at the ninth amino acid. This insert includes 85 bases upstream of the mutation and 613 bases downstream of the mutation, which is more than the 63 bases required in our prior experiments.

We attempted to create both a single mutant and a quadruple mutant of $44^{-}50^{-}51^{-}25.1^{-}$. Because of the homology between 44 and 51 and the similarities in the expected functions (discussed in Ch 1), we wanted to see the effect of mutating all 4 genes. In order to create the quadruple mutant, Aruna Sampath's $44^{-}50^{-}51^{-}$ knockout strain was used to infect CB313(p25.1MutA). To create a single mutant, wild type lysate was used to infect CB313(p25.1MutA). The first recombination attempt for the single mutant was performed by Sean Murray and no MOI was recorded. I then

screened approximately 10,000 plaques from this recombination, with no mutant found. For the quadruple mutant, the recombination experiment had an MOI of 0.4. As with the single mutant, approximately 10,000 plaques were screened with no mutants detected.

5.4 Ability of probes 25.1mutA and 25.1wtA to distinguish mutant and wild type DNA

In order to show that the probes were capable of distinguishing between mutant and wild type DNA, cultures of CB313(p25.1mutA) were plated, as were wild type plaques. The colonies and plaques were then lifted using the plaque lift protocol in Ch 2.10 and probed as described in Ch 5.1. While preferably, this experiment would be performed with both wild type and mutant in bacteria, the *E. coli* cultures containing the plasmid were not stored properly and were therefore unavailable for this experiment. Figure 5.4 shows that the wild type probe [25.1(8377)wt] produced a strong signal when probing wild type plaques, but a weak signal when probing colonies with the mutant plasmid. Considering the increased signal normally noted when colony lifts are performed, this shows a very strong ability to distinguish wild type from mutant. The mutant probe [25.1(8377)mut] had strong binding to the colonies containing the

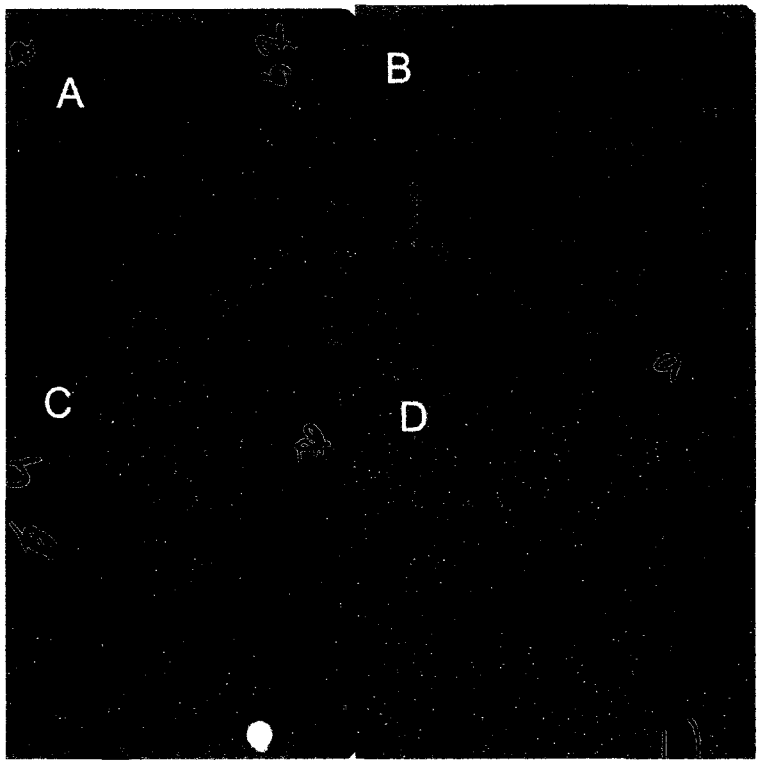

Figure 5.4 Probes 25.1mutA and 25.1wtA: This is a film exposed to membranes A-D. A and C are probed colony lifts from CB313(pTY25.1mutA). B and D are probed plaque lifts of wt SPO1. A, and D were probed with 25.1 (8337)mut, while B and C were probed with 25.1(8337)wt.

mutant plasmid, but essentially no binding to the wild type plaques. This shows that 25.1(8377)wt is capable of binding to the mutant DNA. Since both membranes from wild type plaque lifts were exposed the same amount of time and the 25.1(8377)wt probe versus wild type plaques produced a strong signal while the 25.1(8377)mut probe versus wild type

plaques did not, it is likely that the mutant probes are capable of distinguishing effectively between mutant and wild type.

5.5 p25.1mutB: Recombination Attempt

Since we were unable to find a mutant using the p25.1*mutA* plasmid, we created a new plasmid, with a slightly different mutation (see figure 5.3). Like the initial plasmid, the new wild type plasmid (p25.1wtB) consisted of the full 25.1 gene inserted into pPW19. However, for p25.1wtB, the insert is in the unexpressed orientation. For this plasmid, 89 bases upstream of the gene were also included. While the -10 promoter site was included, the -35 site was not. This is sufficient to prevent gene expression in the bacterial cell. p25.1wtB was mutagenized using the Stratagene Quick Change Site-Directed Mutagenesis Kit, following the provided protocol. The primers 25.1TAA and its reverse complement 25.1TAARC were used, creating a stop codon TAA instead of a lysine (AAG) at the 35^{th} amino acid. This provides 191 bases upstream of the mutation and 533 bases downstream of the mutation for crossing over to occur. This plasmid (p25.1mutB) was believed to have a higher chance of creating a mutant due to the additional bases. Furthermore, the TAA mutation is established in the literature to be well suppressed in CB313. While we believe that the

TAG mutation is also suppressed in this strain, this is not an established fact in the literature. By changing which codon we mutated we hoped that the mutation would be more strongly suppressed and therefore more likely to survive.

Attempts were again made to produce both a single and quadruple (44$^-$50$^-$ 51$^-$25.1$^-$) mutant. For these attempts, the extended, multiple recombination experiment described in Ch5.1.7 was used. For the final round of recombination, the MOI for the wild type phage infection of CB313(p25.1mutB) can not be determined. Plates plated 5 minutes after infection had slightly more colonies (7% more) than those plated immediately prior to infection. The triple mutant infected CB313(p25.1mutB) at an MOI of 1.9. For each lysate, between 10,000 and 12,000 plaques were screened with no positive mutants.

5.6 Ability of probes 25.1mutB and 25.1wtB to distinguish mutant and wild type DNA

With the creation of a new mutant, new probes were also needed. To confirm that these new probes worked effectively, E. coli containing either p25.1mutB or p25.1wtB was plated, colony lifts were performed and the

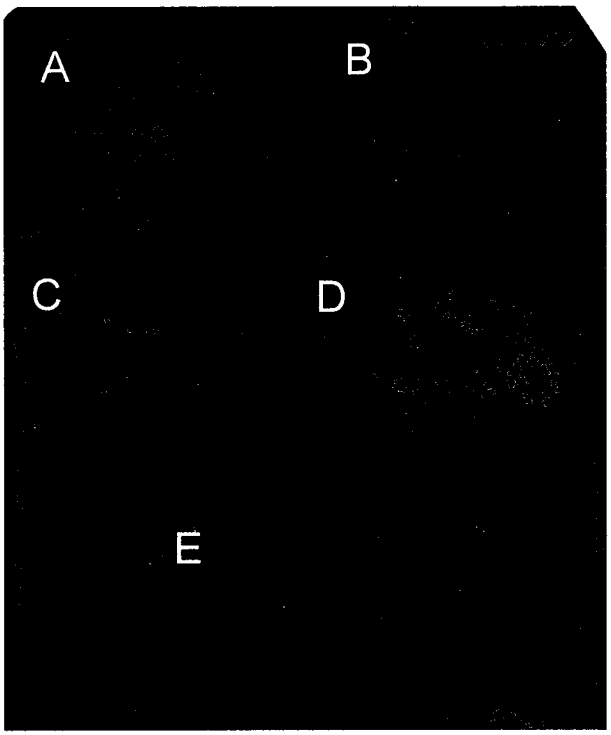

Figure 5.5 Probes 25.1mutB and 25.1wtB: This is a film exposed to membranes A-E. A and B are probed colony lifts from DH5(pTY25.1mutB). C and D are probed colony lifts from DH5(pTY25.1wtB). E is a blank membrane. A,C and E were probed with 25.1 (8449)mut, while B and D were probed with 25.1(8449)wt. Film was exposed to the membranes for 20 minutes.

membranes probed as described above. While colony lifts do routinely give significantly higher signal, two colony lifts from plates with a similar number and size of colonies should yield similar amounts of DNA. Since at this point, the question is whether or not the probes can distinguish between wild type and mutant DNA, as long as the amounts of DNA are similar, the experiment should yield usable results. As seen in figure 5.5 the mutant probe [25.1(8449)mut] creates a strong, consistent signal only when the mutant DNA is present. While some wild type colonies do bind with

25.1(8449)mut probe, the difference in signal is still large enough to distinguish between mutant and wild type DNA. The wild type probe [(25.1(8449)wt] has a strong signal only when probing wild type DNA and almost no signal when probing mutant DNA.

5.7 p2.21mutA: Recombination Attempt

p2.21wtA includes the full 2.21 gene in unexpressed orientation, inserted into pPW19 (see figure 5.3). Only 29 bases upstream of the gene were included. The strongest putative promoter is a middle gene promoter, which would not be expressed from uninfected bacterial cells; however, two putative weak early promoters are also present. While there was no reason to suspect that this gene had a lethal effect, the putative promoters were still not included in order to avoid this potential complication. p2.21wtA was then mutagenized using Stratagene Quick Change Site-Directed Mutagenesis Kit, following the provided protocol. The primer 2.21TAGmut and its reverse complement 2.21TAGRC were used, creating a stop codon (TAG) instead of a lysine (AAA) at the 14^{th} amino acid. This insert included 61 bases upstream of the mutation and 675 bases downstream of the mutation. The plasmid was designated p2.21mutA.

The recombination experiment was performed as described in Ch5.1, with only one round of recombination. In the first recombination experiment, the wild type phage infected CB313(p2.21mutA) with an MOI of 1.5. Approximately 5000 plaques were screened, with no mutants found. The recombination experiment was repeated, this time with an MOI of 1. 10,000 plaques from this lysate were screened with no mutants discovered.

5.8 Ability of probes 2.21mutA and 2.21wt to distinguish mutant and wild type DNA

Plates of E. coli containing either plasmid p2.21mutA or p2.21wtA were grown. Colony lifts were performed as described above and the filters probed with either wild type probe [2.21(5393)wt] or mutant probe [2.21(5393)mut]. As shown in figure 5.6, these probes can distinguish effectively between probe and wild type as strong signal occurs only when 2.21(5393)mut probes mutant colony lifts and 2.21(5393)wt probes wild type colony lifts.

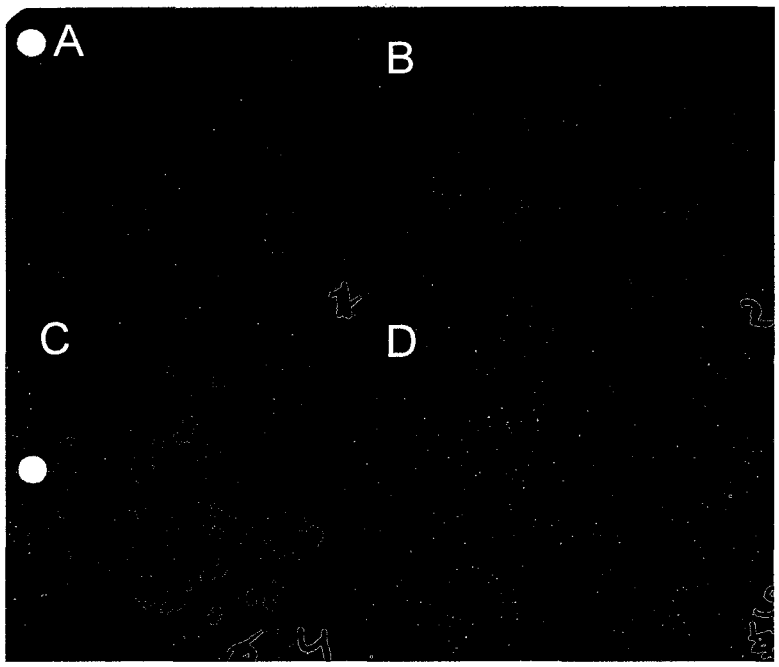

Figure 5.6 Probes 2.21mutA and 2.21wtA: This is a film exposed to membranes A-D. A and B are probed colony lifts from DH5(pTY2.21mutA). C and D are probed colony lifts from DH5(pTY2.21wtA). B, and D were probed with 2.21 (5393)mut, while A and C were probed with 2.21(5393)wt.

5.9 p2.21mutB Recombination Attempts

After failing to find a positive mutant, p2.21wtB was created by inserting the complete 2.21 gene in the unexpressed orientation into pPW19 (see figure 5.3). Additional bases upstream of the gene, including all three putative promoters were included in this plasmid. Since we were able to clone the gene successfully, this indicates that gene 2.21 either is not

lethal or the two early putative promoters are too weak to produce enough of the gene product to kill the cell. Primers 2.21TAA and its reverse complement 2.21TAARC were used to mutagenize the gene using the Stratagene Quick Change Site Directed Mutagenesis kit. The mutagenized plasmid, p2.21mutB, contains 360 bases upstream of the mutation and 698 bases downstream of the mutation.

CB313 containing p2.21mutB was infected with wild type phage, following the multiple recombination protocol defined in Ch5.1. The MOI for the final round of recombination was 1.6. Approximately 11,000 plaques were unsuccessfully screened.

5.10 Ability of probes 2.21mutB's and 2.21wtB to distinguish mutant and wild type DNA

The same protocol for testing the probes for the first 2.21 mutant was used to test the new probes effectiveness. E. coli containing plasmid p2.21mtB or p2.21wtB were plated and probed with either [2.21(5360)mut] or [2.21(5360)wt]. As shown in figure 5.7, the mutant probe [2.21(5360)mut] was effective in distinguishing mutant from wild type, producing a strong signal when probing mutant DNA and almost no signal

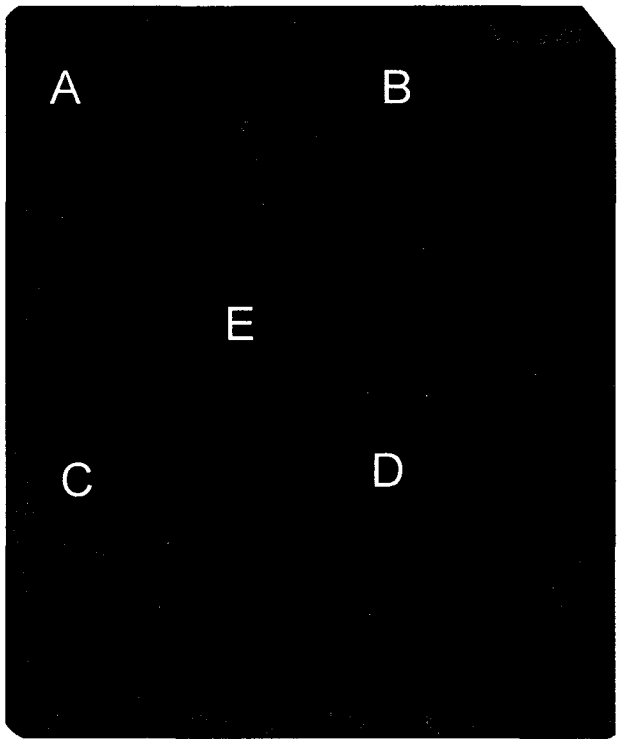

Figure 5.7 Probes 2.21mutB and 2.21wtB: This is a film exposed to membranes A-E. A and B are probed colony lifts from DH5(pTY2.21mutB). C and D are probed colony lifts from DH5(pTY2.21wtB). B and D were probed with 2.21 (5360)mut, while A and C were probed with 2.21(5360)wt. Film was exposed to the membranes for 20 minutes.

when probing wild type DNA. Unfortunately, the wild type probe [2.21(5360)wt] did not distinguish between mutant and wild type DNA, producing strong signal when probing both mutant and wild type colony lifts. However, the wild type probe is not necessary for the mutant screen, so the screening was performed as planned.

5.11 Overview of Results

For all three mutants (25.1^-, 2.21^-, $44^-50^-51^-25.1^-$) desired, two different constructs were attempted, with over 10,000 plaques screened for each. Since we expected 1 mutant for every 1000 wild type plaques, 10 mutant

plaques would have been expected for each mutant. Since we instead found none, there must be an explanation for the lack of mutations. It is possible that our expectation of 1 mutant in 1000 is inaccurate. Both of the genes we attempted to mutate are not in the terminal redundancy. Our expectation of 1 mutant in a 1000 comes from prior work mutating genes in the terminal redundancy. It is possible that the region of DNA containing genes 25.1 and 2.21 have a different, lower frequency of mutation than the terminal redundancy. If the frequency of mutation was 1 in 10,000, missing the one positive plaque is much more likely than missing 10 positive plaques. We had no control to ensure that homologous recombination was occurring at all in our experiment, so it is possible that the rate is zero.

Another possible explanation for the lack of mutants could be that these genes are essential genes. While CB313 does suppress the mutation, it does not fully eliminate it. So, while the gp will still be produced, it will not be produced at wild type levels. If the amount of gene product produced falls beneath the amount required for successful phage infection, the mutant plaque will never appear in a screening. Since this was not a problem when mutating genes 44 and 51, we did not expect this to be a problem for gene 25.1. For gene 2.21, we expected that even with

decreased level of the sigma factor, the infection would still be able to occur. If the plaque is extremely unhealthy and small, it could also be overlooked. However, during our screening, we were extremely inclusive in putative mutant selection in order to minimize this possibility.

A third explanation is that the mutation selection process was flawed. Ideally, the controls limit the possibility of total experimental failure. The probes are clearly capable of binding to the mutant or wild type DNA. Plaque lifts are transferring DNA, which can be detected with wild type probes. The only major factor not controlled for would be that the mutant probes, and only the mutant probes, can not bind to mutant DNA from plaques. This is unlikely since there is nothing distinctly different between the mutant and wild type probes to explain why the mutant probes would not be able to bind to plaque DNA when they could bind to the mutant in the plasmid.

5.12 Future Work

In the future, different methods of mutant construction should be attempted. For example, instead of trying to create a conditional mutant, the entire gene could be knocked out. If the gene was replaced with GFP,

this would be easier to screen for. If the mutation is required for infection to occur, an additional expression vector could be added to the *B. subtilis* strain during production and maintenance of the mutant strain. Gene expression would be induced at the same time as infection. While gene expression may be lethal, during infection the cell is already exposed to many lethal gene products.

Another avenue of research is examining the effect of gps 2.21 and 25.1 on an in vitro transcription/translation system. For this, the gene products must be purified. They could then be added to the in vitro transcription system and the effects monitored. By using promoters specific to immediate and delayed early genes, the effect of regulation on specific gene types can be measured through the in vitro transcription system. Different sigma factors and potential regulators could also be tested. Binding assays looking at whether or not the gene products bind to RNAP specifically could be performed as well. This approach would provide significant information about the gene products function without the need for a mutant.

Ch 6: References

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